IRON(II) INHIBITION OF FILAMENTOUS BACTERIA: ROLE OF TOXICITY ASSESSMENT IN SLUDGE BULKING CONTROL

By

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TO MY WIFE
SOON-HAE LEE

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The subject research was divided into four main parts. In the first portion of the research, several aspects of dehydrogenase assay on activated sludge and filamentous microorganisms using the redox dye 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) were examined. Dimethylsulfoxide (DMSO) outperformed 2+3 tetrachloroethylene/acetone and methanol for extraction of INT-formazan from INT-treated samples. Blank corrections were significant when samples initially treated with ferrous sulfate were assayed. Addition of 0.5 ml of 2.4 N HCl to the 5.5-ml INT reaction mixture was the best means of terminating the enzymatic reaction, but did not prevent changes in formazan yield after prolonged storage of the reaction mixture at 4°C.

In the second portion of the research, the validity of INT-dehydrogenase test was estabilished for assessing toxic inhibition

of filamentous pure cultures (Sphaerotilus natans and type 021N and Thiothrix) and activated sludge by ferrous sulfate. The inhibitory effect on S. natans by ferrous sulfate dramatically increased as pH was changed from 7.0 to 6.0. The order of sensitivity of pure cultures to ferrous sulfate was Thiothrix, Type 021N, and S. natans (from most to least sensitive) in terms of inhibitor concentration for 50% reduction of activity (IC_{50}).

In the third portion of the research, the INT-dehydrogenase activity by counting technique (DHA_c) was used both to predict the iron dose needed to control bulking and to monitor the effect of iron addition of filament activity in a bench scale pilot plant. Successful control of bulking was achieved by adding ferrous sulfate (one third of IC_{50}, and exposure frequency = 3.0 day^{-1}) into the aeration basin during the 4-5 consecutive days until DHA_c fell below 10%.

In the fourth portion of the research, the influence of total suspended solids concentration on iron toxicity to bulking activated sludge was determined. The ferrous sulfate dose required to reduce 50% of filamentous biomass activity increased in proportion to TSS concentration. However, there was no consistent relationship exhibited between the mass based IC_{50} and the suspended solids concentration. Values of IC_{50} for filamentous biomass were always lower than those for gross biomass.

CHAPTER 1
INTRODUCTION

The activated sludge process is popular for secondary wastewater treatment because of its efficiency, flexibility and low land requirements. Operation of activated sludge processes is impaired by the excessive growth of filamentous microorganisms, a phenomenon referred to as bulking. Because of its lower settling and compaction rates, bulking sludge is difficult to separate and compact in secondary sedimentation tanks. Occurrences of activated sludge bulking are common in activated sludge systems. Sometimes much of the activated sludge inventory can be lost over the effluent weir of the final settling tank leading to reduced efficiency or failure of the treatment system.

A rational approach to the prevention or cure of activated sludge bulking is to identify the filament types present and, based on previously established correlations between these types and wastewater composition or operational deficiencies, take actions to correct the cause(s) of filament proliferation. This approach has certain limitations, however. First of all, the techniques used to distinguish filament types are quite subtle in many cases, so that a strong microbiology background and considerable experience are required of those making the

identifications before reliable results can be obtained. Second, the associations between filament types and operational problems or waste characteristics are not always straightforward. For example, a single filament type may be associated with more than one deficiency. Finally, plant flow schemes and operational capabilities may be too inflexible to allow rapid implementation of appropriate remedies even in cases where specific causes of bulking can be identified.

Where sludge bulking is an intermittent or seasonal problem, addition of chemicals such as chlorine or hydrogen peroxide to the aeration tank or to the return sludge may be a more effective approach to bulking control than operational changes or wastewater modification. In any case, short term control of bulking with chemicals may be required while longer term control measures are implemented. The oxidants chlorine and hydrogen peroxide, though widely used to selectively kill filamentous microorganisms in activated sludge, are not without their problems. These chemicals destroy cell integrity through denaturation of protein and other cellular components. Impatience on the part of the operator can lead to overdosage, causing a turbid effluent (Smith and Purdy, 1936; Tapelshay 1945) and possibly destruction of the entire sludge inventory. Besides the oxidants chlorine and hydrogen peroxide, iron salts have also been found effective in bulking control. Waitz and Lackey (1959) observed that growth of Sphaerotilus was inhibited by a concentration of 25 g/m^3 (as Fe)

of ferric chloride. The idea of using iron salts to control sludge bulking was further considered by Pfeffer (1967) and substantiated by other researchers (Carter and Mckinney, 1973; Rensink, 1979). Chang et al. (1979) surveyed the inhibitory effect of selected iron compounds on Sphaerotilus. They reported that soluble iron compounds such as Fe-cystine and ferrous sulfate were highly effective in inhibiting the growth of Sphaerotilus.

The inhibition of iron salts may enable them to kill filamentous microorganisms more selectively than chlorine or hydrogen peroxide and with less chance of damaging floc-forming microorganisms. It has been shown that soluble iron species such as Fe^{2+} are adsorbed to microbial cell surfaces, blocking cell metabolism, in contrast to chlorine and hydrogen peroxide, which destroy cell integrity. Thus, iron salts may provide a safer, easier means to use for bulking control.

The overall objectives of this research were to investigate the effect of iron salts on filamentous bacteria, both in pure culture and in activated sludge, and relate this effect to the toxic inhibition of filament activity. Ferrous sulfate was selected as the model iron compound based on its solubility, availability and relatively low cost.

Specific objectives in this research were as follows:

1. Optimize the INT-dehydrogenase test considering correction for abiotic INT reduction, alternative solvents for extracting INT-formazan, use of enhancers

in the reaction mixture, termination of the enzymatic reaction and preservation of INT-treated samples.

2. Establish the validity of the INT-dehydrogenase test for assessing toxic inhibition of filamentous bacteria in pure culture and in activated sludge following addition of ferrous sulfate by comparing it with oxygen uptake rate and ATP.
3. Use the INT assay to predict the dosage of ferrous sulfate which would be effective in controlling bulking, compare the inhibition of filamentous organisms to inhibition of overall sludge biomass caused by the toxicant, and relate trends in specific activity of filaments to trends in SVI.
4. Determine the effect of total suspended solids concentration on the toxicity of ferrous sulfate to bulking activated sludge.

CHAPTER 2
LITERATURE REVIEW

2.1. The Activated Sludge Process

The activated sludge process was developed in England in 1914 by Arden and Lockett (1914) and was so named because it involved the production of an active mass of microorganisms capable of aerobically stabilizing a waste. The activated sludge process has become more popular than trickling filters because it produces a higher-quality effluent at reasonable cost and offers more flexibility in replacement of accesories and extension (Rensink, 1974). Many versions of the original process are in use today, but they are all fundamentally similar (Metcalf and Eddy, 1979). Biological wastewater treatment with the activated sludge process requires two processing units. The reactor train consists of a single aeration basin or a series of tanks designed to promote specific biochemical reactions. A densely concentrated and largely bacterial culture is maintained in the reactors. The settling tank provides a quiescent environment that allows the activated sludge to separate by flocculation and gravity sedimentation from the treated wastewater. The effectiveness of separation depends on the design and operation of the settling tank and the settling and compaction characteristics of the activated sludge (Pipes, 1979).

For efficient treatment both the aeration basin and the secondary clarifier must function satisfactorily. Therefore factors affecting both biological oxidation and solids separation are important in determining overall process efficiency. Much research and development work have been conducted on biological oxidation processes in activated sludge; however, it is only recently that the factors influencing solids separation have been studied intensively (Jenkins et al., 1984).

2.2. Activated Sludge Bulking

A major problem in the operation of activated sludge processes is caused by the uncontrolled outgrowth of large amounts of filamentous microorganisms leading to activated sludge bulking. Sezgin et al. (1978) postulated that many settling, compaction, and separation properties of activated sludge are related to the relative numbers of filamentous and floc-forming microorganisms in the activated sludge floc. Outgrowth of excessive quantities of filamentous organisms (more than approximately 10 km filaments/g activated sludge dry solids) was correlated with bulking (SVI $\geq$ 150 mL/g).

It has been proposed that the macrostructure of activated sludge flocs is provided by filamentous microorganisms (Sezgin et al., 1978). These organisms form a network or backbone within the floc to which the floc-forming bacteria attach. When an activated sludge culture contains filamentous organisms, large floc sizes are possible because the filamentous organism backbone provides

the floc with strength so that it can hold together in the turbulent environment of the aeration basin. Large flocs containing filamentous organisms tend to become irregularly shaped rather than approximately spherical (as when filamentous organisms are absent or present in small numbers). The filamentous organism network influences the shape of the floc because the floc grows in the directions that the filamentous network grows. Bulking is a macrostructure failure in which filamentous organisms providing the macrostructure are present in large numbers. They interfere with the compaction and settling of the activated sludge either by producing a very diffuse floc structure ("stretched out floc") or by growing in profusion beyond the confines of the activated sludge floc into the bulk medium and bridging between flocs. The type of compaction and settling interference depends on the causative filamentous organism. Table 2-1 indicates the type of settling interference caused by various filamentous organisms observed in activated sludge (Jenkins et al., 1984). With filamentous bulking the sludge particles appear to be similar to normal sludge except that filaments extending from the flocs are much more numerous and much longer. A filamentous bulking sludge settles very slowly and has a high SVI, although the supernatant above the sludge is usually very clear. In certain instances, the sludge may be composed almost entirely of filaments. Then, the effluent from the secondary sedimentation tanks contains an excessive amount of suspended matter and it produces a diluted

Table 2-1. Type of compaction and settling interferences caused by various filamentous organisms. (After Jenkins et al., 1984)

Bridging	Open floc structure
type 021N	type 1701
Sphaerotilus natans	type 0041
type 0961	Microthrix parvicella
type 0803	type 0675
Thiothrix sp.	Nostocoida limicola
type 0041	
Haliscomennobacter hydrossis	

return sludge. Sometimes much of the activated sludge inventory can be lost over the effluent weir of the final settling tank leading to failure of the treatment system.

Occurrences of activated sludge bulking are not uncommon in the activated sludge systems. Surveys undertaken in the early 1940s revealed that approximately two thirds of U.S. plants experienced problems with bulking (Anon, 1943; Dreir, 1945). In Germany, at least 70% of the 108 activated sludge plants surveyed showed SVI greater than 150 mL/g for at least 2 months of the year (Wagner, 1982). Tomlinson (1976) investigated the extent of the problem of bulking sludge in major activated-sludge plants in England and Wales. Fifty two percent of the plants included in the survey experienced bulking to the extent that a serious loss of solids in the final effluent occurred, whereas 41% had yearly average SVI values greater than 200 mL/g. Taking both these conditions together it may also be shown that 63% of the plants experienced either serious losses of solids due to bulking or had SVI values greater than 200 mL/g.

2.3. Filamentous Bacteria in Activated Sludge and Conditions Associated with Their Proliferation

Observed and recorded filament characteristics are used to characterize the filamentous organisms to genus or to a numbered type using the dichotomous key shown in the manual prepared by Jenkins et al. (1984). This key lists the 22 filamentous bacteria most commonly observed in activated sludge. This dichotomous key is a modification of the filamentous organism identification key

given by Eikelboom and van Buijsen (1981).

Surveys were conducted in the USA using Eikelboom's filamentous organism identification procedures and key to examine the relative occurrence of various filamentous organism types in bulking activated sludge. As shown in Table 2-2, Nocardia spp. were the most dominant filaments; however, this microorganism causes foaming rather than bulking. Type 1701 was the second most common filament found. Type 021N and Thiothrix spp. were the 3rd and 4th most common filament types causing bulking, respectively. S. natans, the filament so often cited in the literature, was the 6th most dominant filament. These surveys demonstrate that approximately 10 types of filaments account for over 90% of all bulking episodes. The differences between occurrence in the USA and in South Africa are shown in Table 2-2. In South African plants low F/M conditions are the more likely cause as most activated sludge plants are operated at long sludge ages. In the USA, activated-sludge processes are operated under a wide range of conditions from high-rate (short sludge age, high F/M, possibly low DO) to low-rate (long sludge age, low F/M) (Blackbeard et al., 1986). In Table 2-2 the principal dominant filament types causing bulking in Europe and Germany have been identified in independent surveys by Eikelboom and Wagner, respectively. M. parvicella, type 0041, type 021N and S. natans were found in top seven in the USA, in Europe and in Germany. The differences are for Nocardia spp. (Rank 1 versus 14) and M. parvicella (Rank 7 versus 1)

Table 2-2. Ranking of dominant filament types in South African, European and North American plants. (After Blackbeard et al., 1986)

Filamentous microorganisms	South Africa	USA	Europe	Germany
type 0092	1	11	4	-
M. parvicella	2	7	1	2
type 1851	3	9	12	-
type 0675	4	6	-	-
type 0914	5	-	-	-
type 0041	6	5	6	3
Nocardia spp.	6	1	14	5
type 0803	7	7	9	10
type 1701	8	2	5	8
N. limicola	8	8	11	7
type 021N	-	3	2	1
H. hydrossis	9	9	3	6
S. natans	-	6	7	4
Thiothrix spp.	9	4	17	-
type 0581	9	12	8	-
type 0961	-	10	10	9
Beggiatoa spp.	-	12	-	-
Number of plants	56	190	200	315
Number of samples	56	300	1100	3500

between in the USA and in Europe.

For the latter type, the lower organic loading in Dutch plants, where many oxidation ditches are used, as compared to American plants, provides a probable explanation. With Nocardia spp., the difference in reported prevalence at first suggested the possibility of some difference in wastewater composition or treatment between Europe and the U.S. However, Eikelboom frequently observed Nocardia spp. but deemphasized it because they did not associate it with bulking (Strom and Jenkins, 1984).

The major suggested causes of filamentous bulking (Table 2-3) include aeration basin dissolved oxygen (DO) concentration too low for the applied organic loading, low F/M in completely-mixed, continuously-fed systems, septic wastes, nutrient deficiency (principally nitrogen and/or phosphorous), and low pH (pH < 6.5). Activated sludge treating septic wastes can bulk due to the growth of Thiothrix spp. and type 021N. Recent work has indicated that these filamentous organisms may be favored by septic wastes because they can utilize as energy sources for growth both inorganic, reduced sulfur compounds (e.g. H_2S) and the low molecular weight organic acids (e.g. acetic acid) produced by fermentation processes taking place in septic sewage (Richard et al., 1983). When growing under these conditions these organisms deposit intracellular sulfur granules. The observation of Thiothrix spp. or type 021N in abundant quantities and containing substantial amounts of intracellular sulfur granules therefore may

Table 2-3. Dominant filament types as indicators of conditions causing activated sludge bulking. (After Jenkins et al., 1984)

Suggested causative conditions	Indicative filament types
Low DO	type 1701, S. natans, H. hydrossis
Low F/M	M. parvicella, H. hydrossis, Nocardia sp. types 021N, 0041, 0675, 0092, 0581, 0961, 0803
Septic wastewater/sulfide	Thiothrix sp., Beggiatoa, type 021N
Nutrient deficiency	Thiothrix sp., S. natans, type 021N, and possibly H. hydrossis and types 0041 and 0675
Low pH	fungi

suggest that septic wastewater is the cause of bulking. Type 1701 and S. natans occur when the aeration basin DO is too low for the applied F/M. It is possible that type 1701 may occur at a more severe DO limitation than S. natans (Richard et al., 1982).

2.4. Potential of Iron Salts for Control of Sludge Bulking

2.4.1. Experience with Iron Salts for Bulking Control

In laboratory studies, the addition of ferrous sulfate to the aeration tank has been reported not only to reduce sludge bulking, but also to increase the organic removal efficiency (Pfeffer, 1967; Carter and McKinney, 1973). Waitz and Lackey (1959) observed that growth of Sphaerotilus was inhibited by a concentration of 25 g/m^3 (as Fe) of ferric chloride. The idea of using iron salts to control sludge bulking was further considered by Pfeffer (1967). He found that adding iron and manganese to an activated sludge unit inhibited the growth of the fungi Fusarium and Geotrichum and produced a sludge with good settling characteristics. In a later study, Carter and McKinney (1973) indicated that by supplying an adequate amount of iron (ferric chloride) to the activated sludge system it was possible not only to reduce sludge bulking problems, but also to achieve a higher rate of metabolism. However, too high a concentration of iron had an adverse effect on the normal bacteria. Increasing the amount of ferric chloride added to glucose-fed activated sludge from 2 g/m^3 to 10 g/m^3 increased the rate of metabolism in the activated sludge system, whereas adding 20 g/m^3 of iron had an adverse

effect (Carter and McKinney, 1973). These results reveal that a somewhat enhanced concentration of iron stimulates the growth of normal organisms and inhibits the growth of filamentous organisms but that a high concentration of iron inhibits the growth of both.

Many factors such as the organic content of the substrate, the form of iron compound employed, the pH, and the concentration of organisms in the activated sludge can influence the inhibitory effect of iron on filamentous organisms. Furthermore, the tolerable range for iron may be different for the fungi Fusarium and Geotrichum than for the different types of filamentous bacteria.

2.4.2. Mechanism of Iron Inhibition

Pfeffer and Chang (1977) found that adsorption of iron on Sphaerotilus was the major inhibitory mechanism. Chang et al. (1979) surveyed the inhibitory effect of selected iron compounds on Sphaerotilus. They reported that soluble iron compounds such as Fe-cystine and ferrous sulfate were highly effective in inhibiting the growth of Sphaerotilus and hence were candidate compounds for controlling Sphaerotilus bulking. Although Fe-cystine had the higher inhibitory value, ferrous sulfate has greater practical potential from an economic point of view (Chang, 1979). One of the least costly sources of ferrous sulfate is the waste pickle liquor from steel plants. Chang et al. (1979) showed that both soluble iron and iron precipitate are adsorbed to S. natans. After 24 hours of incubation with iron precipitates, the

inhibited cultures were examined microscopically (Chang, 1979). The organisms in the cultures inhibited with aged $Fe(OH)_3$ were coated with a layer of $Fe(OH)_3$ particles outside their sheath. The particles were approximately 0.1 μm in diameter. The thickness of the coating varied, and parts of the organism were not coated at all. The cultures treated with fresh $Fe(OH)_3$ and $FeCO_3$ were similar to each other in morphology. The particles had been condensed to flocs, and large parts of the organism were not coated with the particles. The cultures treated with 20 g/m^3 of iron-citrate (a soluble iron compound) as Fe were examined microscopically with the aid of the prussian blue staining technique. The organisms in the treated cultures were stained a uniform blue color, indicating the presence of a layer of ferric iron on the organisms. Insoluble iron compounds, such as iron hydroxides and ferrous carbonate, were found to be less toxic to S. natans than soluble iron compounds (iron citrate, iron cystine, and ferrous sulfate).

Chang et al. (1980) reported that there was no correlation between the degree of inhibition and the total amount of iron sorbed by the organisms or the iron sorbed by the sheaths utilizing the method described by Romano and Peloquin (1963). There appeared to be a strong correlation between the amount of iron sorbed by the cells and inhibition. It appears that the site of inhibition is at the cell wall rather than at the sheath. Therefore, for iron to be an effective inhibitor, it must be in a

form that can penetrate the sheath and contact the cells. Sheaths act as protective barriers that prevent the iron from contacting cells. This is substantiated by Pringsheim (1949) and by Mulder and van Veen (1963). They reported that sheaths of Sphaerotilus organisms were coated with a layer of ferric iron precipitate when the organisms were cultured in an iron enriched medium.

The inhibitory effects of iron were proportional to the amount of the sorbed iron on the organisms at low levels of iron concentration. Beyond a certain point, no further increase in inhibition with increasing adsorption was observed (Chang et al. 1980). This could be supported by the effect of reaction time on the sorption of iron in a batch of Sphaerotilus culture to which had been added iron-organic complexes to a concentration of 20 g/m^3 as Fe determined by Chang (1979). The sorption was rapid with the iron cystine complex. With an organism concentration of 105 g/m^3 and an initial iron complex concentration of 20 g/m^3 as Fe, 80 percent of the reaction was complete within 5 min. After approximately two hours, no further reaction was observed. The inhibitory mechanism for a substantial effect of iron on the cells of filamentous microorganisms would be biological rather than physico-chemical in nature. However there are several physicochemical factors to be considered in controlling bulking sludge in terms of available soluble iron that is eventually effective in inhibiting filamentous microorganisms. These are the pH and alkalinity of medium, interactions of iron with inorganics

such as sulfide and phosphate, or organics, and oxidation of Fe(II).

2.4.3. Chemistry of Iron in Water

Themodynamic considerations are useful for obtaining a general understanding of the potential reactions of iron. We can define a convenient parameter for the redox intensity in the same manner for pH.

$$p\epsilon = -\log[e] \qquad (2\text{-}1)$$

where $p\epsilon$ gives the electron activity at equilibrium and measures the relative tendency of a solution to accept or transfer electrons. The following equation is a general expression for the thermodynamic reactions in aqueous solutions. It can be derived based on thermodynamic reactions in aqueous solutions, as shown in Table 2-4.

$$p\epsilon = p\epsilon^{o} + (1/n)\log [Fe^{3+}]/[Fe^{2+}] \qquad (2\text{-}2)$$

where n represents the number of electrons transferred for the general case. The quantity $p\epsilon^{o}$ is the relative electron activity when all species other than electrons are at unit activity. A high $p\epsilon$ indicates a relatively high tendency for oxidation. Under aerobic conditions and neutral pH, $Fe(OH)_3$ is the dominant form in the light of presently available thermodynamic information given by the $p\epsilon$-pH diagram shown in Figure 2-1.

Ferrous iron salts, when added to water, readily precipitate as hydroxides or, if carbonate ions are available, as carbonate. In the absence of dissolved CO_2, the solubility of Fe(II) is

Table 2-4. Derivation of general expression for the iron-thermodynamic reactions in aqueous solution. (After Stumm and Morgan, 1980)

Redox reaction: $Fe^{3+} + 1/2H_2(g) = Fe^{2+} + H^+$ K_1 (1)

where $K_1 = \{Fe^{2+}\}\{H+\}/\{Fe^{3+}\}\{H_2\}_{(g)}^{1/2}$

Reaction (1) is composed of two steps.

$$Fe^{3+} + e = Fe^{2+} \qquad K2 \quad (2)$$

$$1/2H_2(g) = H+ + e \qquad K3 \quad (3)$$

According to thermodynamic concentration: $K_3 = 1$

Thus $K_1 = K2K3 = \{Fe^{2+}\}/\{Fe^{3+}\}\{e\}$ K4 (4)

Take negative log both side of eqn. (4)

$$p\epsilon = p\epsilon^\circ + \log [\{Fe^{3+}\}/\{Fe^{2+}\}] \qquad (5)$$

Since $\Delta G^\circ = -RT\ln K$

$$\log K = -\Delta G^\circ/2.3RT$$

$$p\epsilon^\circ = \log K = -\Delta G^\circ/2.3RT$$

$$p\epsilon = -\Delta G^\circ/2.3RT + \log[\{Fe^{3+}\}/\{Fe^{2+}\}] \qquad (6)$$

For the general case where n electrons are transferred:

$$ox + ne = red \qquad K^* \quad (7)$$

$$K^* = \{red\}/\{ox\}\{e\}^n$$

$$p\epsilon = \log K^*/n + (1/n)\log[\{ox\}/\{red\}]$$

$$= p\epsilon^\circ + (1/n)\log[\{ox\}/\{red\}] \qquad (8)$$

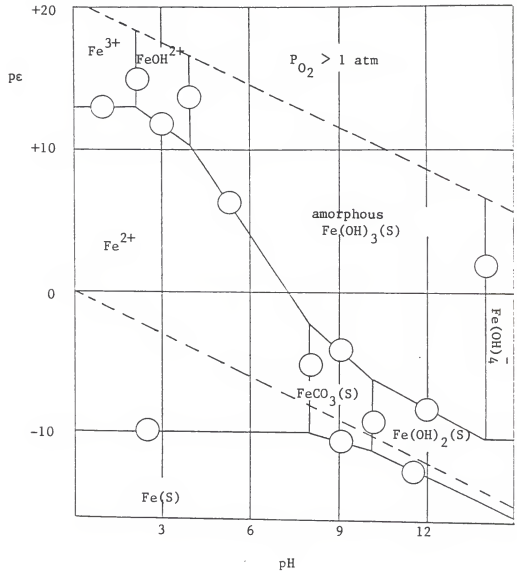


Figure 2-1. pε-pH diagram for the Fe-CO_2-H_2O system (25°C) (After Stumm and Morgan, 1980)

controlled by solid ferrous hydroxide. However, between pH 5.0 and 9.0, ferrous carbonate is the major precipitate when the total carbonate content is higher than 1 mM, a condition commonly found in wastewater. The solubility of ferrous carbonate depends on pH. When ferrous iron salts are added to water under aerobic conditions, ferrous iron is oxidized to ferric iron by oxygen. Ferric iron then precipitates readily as ferric hydroxide. Because the solubility of the ferrous precipitate (ferrous carbonate) is higher than that of ferric hydroxide, it would be desirable to maintain the iron in the ferrous form as long as possible if soluble iron were more effective than iron precipitates in inhibiting filamentous bacteria. Hence the factors that may affect the rate of oxygenation of ferrous iron must be considered.

The rate of oxygenation of Fe(II) in solution of pH > 5 was found to be first-order with respect to concentrations of both Fe(II) and O_2 and second order with respect to the concentration of OH^- ion. Oxidation of Fe(II) is very slow below pH 6 (Stumm and Morgan, 1980). Figure 2-2 shows the oxidation rate of ferrous iron between pH 1-7. At pH values greater than 5.0, the rate law for the oxygenation of ferrous iron is (Stumm and Lee, 1961):

$$-d[Fe(II)]/dt = k\,[Fe(II)]\,[OH^-]^2\,pO_2 \qquad (2\text{-}3)$$

where k = rate constant = 8.0×10^{13} liters^2/atm-min-mole^2 at 25°C, and pO_2 = the partial pressure of oxygen in atmospheres. Below pH 5.0, the rate law appears to follow the form (Singer and

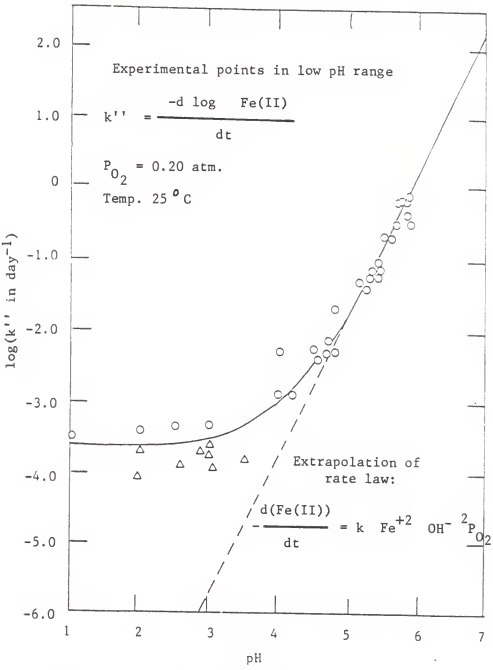


Figure 2-2. Oxidation rate of ferrous iron as a function of pH (After Stumm and Morgan, 1980)

Δ : results obtained by Singer and Stumm (1970)

O : results obtained by Stumm and Lee (1961)

Stumm, 1970).

$$-d[Fe(II)]/dt = k' [Fe(II)] pO_2 \qquad (2\text{-}4)$$

where $k' = 1.0 \times 10^{-7}$ $atm^{-1}min^{-1}$ at 25°C. In the pH range between 6.0 and 7.0, the rate of oxygenation of ferrous iron is rather slow; the half life of ferrous iron under aerobic conditions, as calculated from equation (1), is around 7 hours at a pH of 7.0 and 30 days at a pH of 6.0. Proper control of the pH is thus of considerable importance when using ferrous sulfate to inhibit filamentous bacteria.

Formation of Fe(II) complexes with ortho-phosphate and many organic bases is well established. In order to evaluate the coordinating tendency of Fe(III), the relative affinities of Fe(III) for OH^- ions and other ligands need to be compared. Hydroxide ions often have a stronger affinity for Fe(III) than do organic or inorganic bases. The extent of complex formation is thus pH dependent, and within the pH range of natural waters, soluble or insoluble mixed Fe(III) complexes that may contain OH^- as well as other ligands can be formed. For example, Fe(III) interacts chemically with orthophosphate to form soluble phosphate-iron(III) complexes (e.g., $FeHPO_4$, $Fe_x(H_nPO_4)^{3x-(3-n)y}$). Under slightly acid conditions pure $FePO_4(s)$ will be precipitated, whereas in the neutral and slightly alkaline pH range the precipitate is probably a ferric compound containing both PO_4^{3-} and OH^- in variable proportions, depending on the pH. The chemical interaction of Fe(III) with many other organic bases are

similar to that with orthophosphate.

2.5. The INT-Dehydrogenase Assay and Its Use to Assess Toxic Inhibition of Filamentous Bacteria in Activated Sludge

Addition of toxicants to activated sludge will be effective in the control of bulking if the applied dosage is high enough to kill filaments extending from activated sludge flocs, but not so high as to significantly inhibit the floc formers themselves. In order to apply toxicants properly for bulking control, a rapid and sensitive technique is needed to determine the specific activity of filamentous bacteria in mixed cultures. Such a technique should make it possible to determine the *in situ* resistance of filamentous bacteria in bulking sludge (so that effective toxicant dosage can be predicted) as well as to assess rapidly the effect of toxicant additions on filaments during the period in which treatments are made.

The most direct means of determining the effective dosage is by a toxicity bioassay with the sludge and toxicant in question. There are several techniques to assess the inhibitory effect of toxicants, but until recently no satisfactory assay was available for assessing the inhibitory effect of toxicants specifically on the filaments in the activated sludge. Non-physiological assays (e.g. sludge volume index, morphological observation using phase microscopy) are available for estimating the degree of proliferation of filamentous microorganisms. However, these assays lack sensitivity or are difficult to quantify because of their non-physiological characteristics. However, physiological

assays such as oxygen uptake rate (OUR) and adenosine triphosphate (ATP) cannot differentiate between the activity of filamentous biomass and the gross (overall) activity of activated sludge. Certain redox dyes, including methylene blue (Jorgensen 1984), resazurin (Liu 1983), and various tetrazolium salts (Altman 1976, 1977) can substitute for terminal acceptors in electron transport system (ETS) of active microorganisms. Tetrazolium salts possess the property of being easily reduced to intensely colored, water-insoluble formazans in active cells.

Bitton and Koopman (1982) developed a tetrazolium reduction assay for determining the physiological activity of filamentous microorganisms in activated sludge. The basic technique involves the reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan by active bacterial electron transport systems, as described by Zimmerman (1975). The ETS activity of filamentous bacteria (specific activity, DHA_c) is measured by comparing the total length of active filaments (containing red INT-formazan crystals) to the total length of all (active plus non-active) filaments observed microscopically under bright field illumination. The test also provides for determination of gross dehydrogenase activity (DHA_e) in the same sludge. This is accomplished by extraction and spectrophotometric quantification of the INT-formazan formed during the incubation step (Lopez et al., 1986). An advantage in using INT over methylene blue or 2,3,5-triphenyltetrazolium chloride (TTC) is

that this compound is insensitive to oxygen (Altman 1970), allowing tests to be carried out under aerobic conditions.

Logue et al. (1983) reported that trends in DHA_c of S. natans anticipated similar trends in SVI and that DHA_c allowed accurate and rapid prediction of effective chlorine dosage for filamentous bulking control in an activated sludge pilot plant. Koopman et al. (1984) found that DHA_e was well correlated with dissolved oxygen uptake rate and DHA_c was an accurate predictor of changes in sludge settleability caused by hydrogen peroxide addition. Lopez et al. (1986) investigated the effect of INT dosage, VSS concentration, pH, sludge age, and chlorine stress on DHA_e. They found that DHA_e was directly proportional to INT dosage and inversely proportional to biomass concentration over limited ranges. Good correlations between DHA_e and OUR of chlorine stressed activated sludge were found at sludge ages ranging from 2.2-7.0 d.

CHAPTER 3
EXPERIMENTAL APPROACH AND RESEARCH OBJECTIVES

The INT-dehydrogenase test was selected as a toxicity bioassay to assess the toxic inhibition of filamentous microorganisms in activated sludge as an adjunct to bulking control by ferrous sulfate as discussed in the introduction (chapter 1) and literature review (chapter 2). First, the test was optimized with respect to extraction of INT-formazan by alternative solvents, use of permeabilizing agents to facilitate INT-formazan extraction, correction for abiotic INT reduction, and termination of the enzymatic reaction and preservation of the INT-treated sample. Second, three of the most common filament types in USA plants (type 021N, S. natans and Thiothrix) were selected as test filamentous bacteria. The inhibitory effect of ferrous sulfate on these bacteria in pure culture and in activated sludge was determined using oxygen uptake rate, ATP, and dehydrogenase assay. Third, a study was performed to demonstrate that the INT-dehydrogenase test can be used as an adjunct to sludge bulking. The growth of filamentous bacteria was controlled by appropriate manipulation of organic loading (F/M), dissolved oxygen concentration in the aeration basin and strength of the feed in the bench-scale pilot plant. Fourth, the effect of total

suspended solids concentration on the toxicity of ferrous sulfate to filamentous bacteria in activated sludge was determined using the INT-dehydrogenase test. Table 3-1 provides a summary of experiments.

Table 3-1. Summary of experiments

Objectives	*Experiments
Optimization of INT-dehydrogenase test	a. extraction of INTF by alternative solvents b. absorptivity of INTF c. effects of lysozyme and Triton X-100 d. blank correction for abiotic INT reduction e. termination of INT reduction and preservation of INTF
Assessment of iron toxicity to filamentous bacteria and activated sludge	a. growth of S. natans in CGY medium b. effect of pH on iron toxicity to S. natans c. assessment of iron toxicity to S. natans d. assessment of iron toxicity to type 021N e. assessment of iron toxicity to Thiothrix f. distribution of iron in ferrous sulfate treated Thiothrix g. assessment of iron toxicity to activated sludge
INT-dehydrogenase assay as an adjunt to bulking control with ferrous sulfate	a. experiment 1 a.1. stat up and intial development of bulking sludge a.2. first application of ferrous sulfate a.3. recurrence of bulking and second application of ferrous sulfate b. experiment 2 b.1. start up and development of bulking sludge b.2. first addition of ferrous sulfate b.3. recurrence of bulking and second addition of bulking b.4. final recurrence of bulking
Effect of suspended solids conc. on iron toxicity to bulking activated sludge	a. development of bulking sludge b. effect of SS conc. on iron toxicity to filamentous microorganisms in bulking activated sludge c. effect of SS conc. on iron toxicity to gross biomass in bulking activated sludge

CHAPTER 4
OPTIMIZATION OF THE INT-DEHYDROGENASE TEST ON ACTIVATED SLUDGE AND FILAMENTOUS BACTERIA

4.1. Introduction

The activity of microbial dehydrogenases may be determined either *in vitro* or *in vivo*. The latter method is the more rapid as it avoids the need for an initial step (e.g., grinding or ultrasonication) to release the enzymes. *In vivo* dehydrogenase assay utilizing the redox dye, 2-(*p*-iodophenyl-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT), has been applied to measure microbial activity in natural waters, soils, sediments and actinomycete foams and has also proven useful for assessing the toxicity of heavy metals, organics and oxidants to wastewater bacteria and activated sludge (Zimmermann et al., 1978; Trevors et al., 1982; Zimmerman, 1975; Awong et al., 1985; Dutton et al., 1986; Kim et al., 1987). The validity of dehydrogenase assay is indicated by the highly significant, linear correlations found between dehydrogenase activity, oxygen uptake and adenosine triphosphate in microbial systems (Benefield et al., 1977; Trevors, 1984; Lopez et al., 1986; Awong et al., 1985). Only a few of the procedual variables which influence test results have been investigated, however. These include INT dosage, biomass

concentration and pH (Lopez et al. 1986). The present study considered alternative solvents for extracting INT-formazan, use of permeabilizing agents to facilitate INT-formazan extraction, correction for abiotic INT reduction, termination of the enzymatic reaction and preservation of INT-treated samples.

4.2. Materials and Methods

4.2.1. Microbial Cultures

The primary source of activated sludge samples was a completely mixed process treating domestic and commercial wastewaters from the city of Gainesville (Main St. plant). Additional samples were obtained from a contact stabilization process treating domestic and hospital wastewaters from the University of Florida campus (UF plant). Mean organic loadings at the two facilities were 0.10 and 0.067 grams five-day biochemical oxygen demand daily per gram volatile suspended solids (VSS) under aeration, respectively. Sludges from both sources had low values of sludge volume index (<150 mL/g), indicating that their contents of filamentous microorganisms were relatively small (Lee et al. 1983). Sludge samples were aerated 4-8 hours at 22 $\pm$ 2°C, then diluted to a VSS between 0.6 and 2.0 kg/m^3, using unchlorinated secondary effluent which had been previously filtered through 1.2 μm pore size, glass fibre filters (Whatman GF/C).

Filamentous bacteria used in the pure culture studies were type 1701, type 021N and Sphaerotilus natans, which are the second, third and sixth most abundant filament types observed in

U.S. bulking sludges (Jenkins et al., 1984). Pure cultures were obtained from Dr. Michael Richard, Colorado State University. Type designations refer to the key of Eikelboom (Eikelboom 1975, 1977) as modified by Strom and Jenkins (1984). Liquid cultivation media were glucose mineral salts medium (GMB; Lau et al., 1984) for type 1701, modified glucose mineral salts medium (GMBN; Richard et al., 1985) for type 021N and CGY (Dondero et al., 1961) for S. natans. Cultures were grown in 2-litre Erlenmeyer flasks (1.0 litre liquid volume) on a gyrotory shaker (Lab Line model 3590; 90 oscillations/min) at 24-28°C. Bacteria were harvested after reaching the declining growth phase (5-7 days), which is representative of their state in the activated sludge process (Weddle and Jenkins, 1971). Culture medium was centrifuged at 1500 x g for 15 min and the supernatant discarded. Biomass was resuspended in Tris-HCl buffer (0.05 M, pH 7.6 or as indicated). Total volume was adjusted to give a biomass concentration of 0.3-0.5 kg total suspended solids (TSS)/m^3.

TSS and VSS were determined according to APHA (1980), methods 209D and 209G, respectively. Membrane filters (0.45 μm pore size, Gelman GN6) were used in the TSS measurements on the pure cultures whereas GF/C filters having an effective pore size of 1.2 μm were employed for VSS determinations on activated sludge.

4.2.2. Procedure for INT Reduction Assay

The pH of the bacterial suspensions was adjusted to 7.6 using 0.1 N HCl or 0.1 N NaOH. Then, 0.5 parts of 3.95 mM INT (Eastman

Kodak) were added to 5.0 parts of sample. (Preparation and storage of the INT solution were described previously (Lopez et al., 1986)). In some experiments, 1 part lysozyme or Triton X-100 solution was added to the sample before the INT. Incubation of the reaction mixture was carried out in the dark at 22 ± 2°C in Erlenmeyer flasks or test tubes. The flasks were mixed on a shaker table (Model 3590, Lab Line) at 100 oscillations/min, whereas the test tubes were agitated using a hematology mixer. INT reduction was terminated after exactly 20 min by adding a quench solution (e.g., 1.0 part 37% formaldehyde per 5 parts of sample). From the equations presented by Lopez et al. (1986), the following general expression for calculating INT-dehydrogenase activity was derived:

$$DHA = \frac{16{,}000\ D\ v}{e\ b\ C\ V\ t\ F} \times 1440 \qquad (1)$$

where DHA is expressed in units of equivalent oxygen uptake (mg O_2*/g VSS-d), 16,000 is the mass of O_2 (in mg) equivalent to 1 mol INTF, D = optical density at the wavelength of peak absorption, v = volume of extract (ml), e = extinction coefficient ($M^{-1}cm^{-1}$), b = path length (cm), C = biomass concentration of sample (g VSS/l), V = volume of INT-treated mixture extracted (ml), t = incubation period (min), F = factor to adjust for the dilution of the sample caused by adding INT at the beginning of the incubation period and quench solution at the end of this period, and 1440 converts min^{-1} to d^{-1}.

4.2.3. Experimental Procedures

4.2.3.1. Extraction of INTF by alternative solvents

The solvents tested were dimethylsulfoxide (Fisher, A.C.S.), methanol (Fisher, A.C.S.) and a solution of 2 parts tetrachloroethylene (Fisher, reagent grade) + 3 parts acetone (Fisher, A.C.S.). One to six mL of the quenched reaction mixture (depending on color intensity) was pipetted into a 15 mL centrifuge tube and centrifuged at 1500 x g for 10 min. After carefully decanting the supernatant, 5 mL of one of the solvents was added to the centrifuge tube and the tube capped. The pellet was resuspended by vortexing for 15 s and extraction of INTF allowed to continue in darkness. The extraction times were 30 min for 2+3 tetrachloroethylene/acetone (TA) and methanol and 15 min for dimethylsulfoxide (DMSO). (Extractions with DMSO were visually complete at the shorter time; longer extraction periods resulted in turbid solutions.) The extract was clarified by centrifugation (1500 x g, 10 min) and the optical density of the supernatant determined by absorption spectrophotometry (Baush and Lomb Spectronic 70, 1 cm path length) at the appropriate wavelength. Blanks containing autoclaved samples were carried through the procedure to correct for abiotic INT reduction. The optical density of the blanks ranged from 0.02-0.05, the higher values being associated with extractions by DMSO. Extractions were carried out in triplicate except as noted.

4.2.3.2. Absorptivity of INTF

The optical density of 0.03 mM solutions of INTF reagent (Sigma) and extracts of INT-treated sludge were scanned over a range of wavelengths to give the absorption spectra. Solvents employed were methanol and DMSO. At the wavelength of peak absorption in each solvent, the optical density of a series of INTF standards was determined to give the standard curve.

4.2.3.3. Exposure of samples to ferrous sulfate

Sample volumes of 100 mL in 250-mL Erlenmeyer flasks were amended with appropriate volumes (0.1-1.5 mL) of 18 mM ferrous sulfate to give desired Fe^{2+} dosages. The toxicant stock solution was prepared with $FeSO_4$ $7H_2O$ and stored up to one week at 4°C. Treated aliquots were agitated for 2 hours at 100 oscillations/min before assaying for dehydrogenase activity. Temperature during the contact period was 22 $\pm$ 2°C.

4.2.3.4. Effects of lysozyme and Triton X-100

Working solutions of lysozyme were prepared with Grade I reagent from Sigma (activity = 39,000 units/mg) and 0.05 M Tris (pH 7.6). In the first experiment, lysozyme was added to the sample at the beginning of the incubation period. In the following two experiments, the enzyme was added to reaction mixtures after incubation was complete. Controls received Tris in lieu of lysozyme.

Working solutions of Triton X-100 (Sigma) were prepared using sterile, distilled water. The surfactant was added to sample at

the beginning of the incubation period in the first experiment. Subsequently, it was added to reaction mixtures after incubation was complete. Controls received sterile, distilled water in lieu of Triton X-100. Tubes were vortexed for 5 s after adding the permeabilizing agent or control solution in order to ensure complete dispersion.

4.2.3.5. Blank correction for abiotic INT reduction

A sludge sample was split into two portions, one of which was autoclaved. Each portion was further divided into two 100 mL aliquots, which were contained in foil-wrapped, 250 mL Erlenmeyer flasks. One aliquot containing autoclaved sludge and one containing live sludge were dosed with 10 g Fe^{2+}/m^3. After two hours of mixing at 100 oscill./min, the aliquots were each amended with 10 mL INT. Agitation was then continued for an additional 45 min. Duplicate 5.0 mL subsamples were removed periodically from the INT-treated aliquots, placed in screw-top test tubes, and fixed immediately with 1.0 mL 37% formaldehyde.

4.2.3.6. Termination of INT reduction and preservation of INTF

In the first of two trials, six subsamples of sludge were dosed with ferrous sulfate at concentrations ranging from 0-10 g Fe^{2+}/m^3. Each subsample was divided into 12 tubes and carried through the initial steps of the nominal assay procedure. INT reduction in triplicate tubes was terminated with 1.0 mL 37% formaldehyde (final concentration = 5.7%), 1.0 mL 1+1 37% formaldehyde/1 M H_3PO_4 (pH 2.5, 2.8% HCHO, 0.23N H_3PO_4), 0.1 mL 12

N HCl (0.21N HCl), or 0.5 mL 2.4 N HCl (0.20N HCl). The contents of one tube from each set of triplicates was extracted 2.5 hours after addition of quench solution. Additional extractions were performed twice over a period of 14 days. In the second trial, the S. natans sample was divided into four sets of 8 tubes each, then carried through the initial steps of the nominal assay procedure. INT reduction in each set was terminated with one of the alternative quench solutions. The contents of two tubes from each set were extracted after 3 hours. Additional extractions on duplicate tubes were performed three times over a period of 22 days. In the third trial, variation of INTF yields of activated sludge samples treated with 2.4N HCl was measured during 8 days storage. All measurements were conducted in triplicate and absorbance values were averaged. Changes in the pH of activated sludge samples treated with above alternative quenching solutions were observed during three weeks prolonged storage. Tubes were stored at 4°C in the dark until their contents were extracted. All extractions were carried out using DMSO.

4.3. Results and Discussion

4.3.1. Evaluation of the Formazan Extraction Step

As part of the in vivo assay of INT-dehydrogenase activity, iodonitrotetrazolium formazan (INTF) formed intracellularly via biochemical reduction of INT is usually extracted from microbial cells to facilitate colorimetric quantitation. Solvents employed for INTF extraction have included 2+3 tetrachloroethylene/acetone

(Zimmerman, 1975; Benefield et al., 1977; Lopez et al., 1986), methanol (Trevors et al., 1982) and dimethylsulfoxide (Dutton et al., 1986). The efficiency of extraction will depend in part on the ability of solvents to penetrate the cell wall and cytoplasmic membrane. These cellular components vary in structure and composition among bacterial species (especially between gram positive and gram negative types) and, concomitantly, will have differing permeabilities to solvent and INTF molecules. Other characteristics such as cell size, lipid content and protection by a sheath, as well as the external cell environment, particularly the surfactants present, could exert profound effects on extraction efficiency.

As part of the evaluation of the formazan extraction step, selected samples were treated initially with the coagulating metal salt, ferrous sulfate. This compound was used as a model of surface active agents that tend to adsorb at the cell surface, forming a barrier to solute exchange (Chang et al., 1980). Lysozyme and Triton X-100 were used as models of surface active agents that react with the cell wall or cytoplasmic membrane, increasing their permeability (Ryssov-Nielson, 1975, Lehninger, 1982, Miozzari et al., 1978). The latter two compounds were added to reaction mixtures of INT and biological sample and their effect on subsequent extractions determined.

4.3.1.1. Absorptivity of INT-formazan in alternative solvents

Previous studies have established that peak absorption of light by INT-formazan (INTF) in 2+3 tetrachloroethylene/acetone occurs at a wavelength of 490 nm and the corresponding extinction coefficient (ϵ_{490}) has a value of 22,500 $M^{-1}cm^{-1}$ (Packard and Healy, 1968; Kenner and Ahmed, 1975; Lopez et al., 1986). The light absorption characteristics of INTF in methanol and DMSO as determined in this study are shown in Fig. 4-1. The absorption peak in methanol was at a wavelength of 480 nm whereas the maximum in DMSO occurred at 465 nm (top Fig. 4-1). Absorption peaks at the same wavelengths were reported by Trevors et al. (1982) and Dutton et al. (1986), respectively. It is interesting to note that scans of light absorption by INTF extracted from activated sludge (lines B) exhibited identical shapes as scans of light absorption by Sigma INTF reagent (lines A). Standard curves of optical density at the wavelength of peak absorption versus concentration of Sigma INTF reagent are shown in the bottom portion of Fig. 4-1. The extinction coefficients, as indicated by the slopes of the linear relationships, were 22,600 $M^{-1}cm^{-1}$ in methanol (ϵ_{480}) and 23,000 $M^{-1}cm^{-1}$ in DMSO (ϵ_{465}).

4.3.1.2. Relative extraction efficiency and the effect of ferrous ion

The relative extraction efficiency of the three solvents for activated sludge, before and after treatment with ferrous sulfate, is shown in Table 4-1. On the untreated subsample, the TA, DMSO

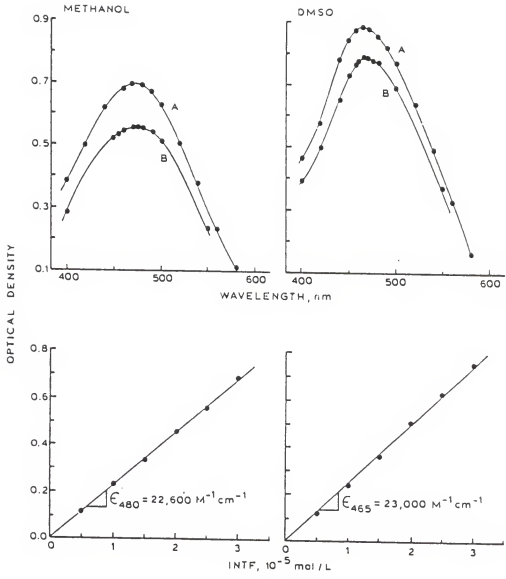


Figure 4-1. Optical properties of INT-formazan in methanol and DMSO

Top-absorption spectra (A = INTF extracted from activated sludge; B = Sigma reagnet grade INTF)
Bottom-standard curves

Table 4-1. Extraction of INTF from activated sludge by alternative solvents

Solvent[a]	Untreated sludge		Treated with 25 g Fe^{2+}/m^3	
	O.D.	INTF, μM	O.D.	INTF, μM
TA	0.697 ± 0.008	31.0 ± 0.34	0.335 ± 0.010	14.9 ± 0.43
DMSO	0.671 ± 0.026	29.2 ± 1.15	0.350 ± 0.011	15.2 ± 0.47
MeOH	0.537 ± 0.011	23.7 ± 0.49	0.208 ± 0.008	9.2 ± 0.38

[a]TA = 2+3 tetrachloroethylene/acetone, DMSO = dimethylsulfoxide, MeOH = methanol

and methanol extracts had optical densities of 0.697, 0.671 and 0.537, respectively (Table 4-1, left). INTF concentrations were determined by dividing these values by the appropriate extinction coefficients. TA yielded a concentration of 31.0 μM, which was slightly greater than the yield of 29.2 μM obtained with DMSO. Both of these values were significantly greater ($P < 0.01$) than the 23.7 μM yield obtained with methanol.

The INTF yields from sludge initially exposed to 25 g/m^3 Fe^{2+} were decreased relative to those from untreated sludge (Table 4-1, right). This effect can be largely attributed to the inhibitory effect of ferrous sulfate on activated sludge (Chang et al., 1980). The apparent inhibition varied with respect to the solvent utilized for extracting INTF, however. With TA the apparent inhibition was 52%, whereas with DMSO it was 48% and with methanol it was 61%. This indicates that extraction with TA and methanol (relative to DMSO) was impaired by the presence of ferrous ion. To further evaluate this effect, INTF yields with TA and methanol were expressed relative to yields with DMSO (Table 4-2). Data from a second experiment are also included in the table. As indicated, ferrous sulfate pretreatment reduced the relative extraction efficiency of TA by 8-17%, whereas the relative efficiency of methanol was reduced by 21-34%.

Results of extractions on filament type 1701 are shown in Table 4-3. This is a gram negative bacterium which has a transparent, closely fitting sheath (Jenkins et al., 1984).

Table 4-2. Effect of ferrous sulfate on relative extraction efficiency of TA and methanol

Solvent	Trial	Relative efficiency,[a] %		Δ effic., %
		Untreated sludge	Sludge treated with 25 g Fe^{2+}/m^3	
TA	1	106 ± 1.2	98 ± 2.8	- 8
	2	106 ± 8.9	89 ± 1.2	- 17
MeOH	1	81 ± 1.7	60 ± 2.5	- 21
	2	96 ± 5.4	62 ± 2.6	- 34

[a]Relative efficiency, % = $\frac{\text{INTF yield with TA or MeOH}}{\text{INTF yield with DMSO}}$ x 100

Table 4-3. Extraction of INTF from filament type 1701 by alternative solvents

Solvent	O.D.	INTF, μM
TA	0.028 ± 0.001	1.2 ± 0.1
DMSO	0.262 ± 0.027	11.4 ± 1.2
MeOH	0.178 ± 0.010	7.9 ± 0.4

Extraction of the INTF crystals by TA was visibly incomplete. After 30 min, it was observed microscopically that the filaments still contained distinct formazan crystals. Extending the extraction time to as long as 24 hours failed to result in complete dissolution of the crystals. The INTF concentration obtained in 30 min with TA was only 1.2 μM. Methanol provided a more complete extraction than TA, but some red color still remained in the filaments after 30 minutes. The INTF yield with methanol was 7.9 μM. The most complete extraction was obtained with DMSO. Dissolution of formazan crystals appeared visually to be complete in 15 min. The INTF concentration obtained in that time was 10.7 μM.

Further comparisons were carried out using samples of S. natans and filament type 021N which had been treated with ferrous sulfate. S. natans has a clear, tightly fitting sheath whereas type 021N, though not sheathed, possesses a heavy cell wall (Jenkins et al., 1984). Both of these organisms are gram negative. A preliminary experiment indicated that methanol was less effective than either TA or DMSO, so the performance of the latter two solvents only is compared here. As shown in Table 4-4, the two solvents gave similar results, although INTF yields with DMSO were generally somewhat greater than yields with TA. (This difference was not significant at $P < 0.01$.) Some color remained in the S. natans cells after extraction by TA, whereas DMSO appeared visually to give a complete extraction. Both

Table 4-4. Extraction of INTF from Fe^{2+}-treated S. natans and filament type 021N by TA and DMSO

Bacteria	$FeSO_4$, g Fe^{2+}/m^3	Contact pH	INTF, μM[a]	
			TA	DMSO
S. natans	20	6.5	30.3	26.7
	20	6.0	5.5	6.7
	20	5.5	2.7	3.9
type 021N	0	7.6	37.0	38.7
	2	7.6	26.0	25.9
	5	7.6	17.5	20.7
	10	7.6	18.8	17.1
	15	7.6	11.1	10.7

[a]Values are the mean results from duplicate extractions

solvents completely decolorized the type 021N bacteria.

4.3.1.3. Permeabilization of cells to improve extraction efficiency

The previous results indicate that DMSO consistently and efficiently extracts INTF from activated sludge and filamentous bacteria. Use of this solvent may not always be possible, however. For example, the color of INTF extracted from activated sludge by DMSO was found to be green instead of red in the presence of some industrial wastes (personal communication with C. L. Logue, Wastewater Branch, Jacksonville, FL, 1986). This problem was not encountered with methanol. It was therefore of interest in the present study to find a means of improving the efficiency of INTF extraction by methanol. Disruption of INTF-containing cells by freezing (Zimmerman, 1975), ultrasonication or french press (Dobrogosz, 1981) may be effective, but would increase substantially the time required for dehydrogenase assay. Alternatively, permeabilizing agents can be added to the reaction mixture and allowed to act during the normal sequence of steps in the assay, thereby not increasing time requirements. Two permeabilizing agents commonly used in bacteriological studies are lysozyme and Triton X-100.

4.3.1.4. Effect of lysozyme

Lysozyme catalyzes the hydrolytic cleavage of complex polysaccharides in the protective cell walls of some bacteria (Lehninger, 1982). Ryssov-Nielson (1975) reported that 0.1 kg/m^3 lysozyme in the reaction mixture of activated sludge and 2,3,5-

triphenyltetrazolium chloride (TTC) improved the reproducibility of the TTC test but not the yield of formazan.

In an initial experiment with samples of activated sludge and filamentous bacteria, 0.07 kg/m^3 lysozyme was added to the reaction mixtures immediately before addition of INT. The subsequent extractions were carried out with DMSO. As shown in Table 4-5, lysozyme had no effect on the yield of INTF from activated sludge, whereas the yield of INTF from S. natans and type 1701 was significantly decreased. The latter result indicated an inhibitory effect of lysozyme on INT reduction by the filamentous bacteria. In subsequent experiments, lysozyme was added after INT reduction had been quenched with formaldehye in order to avoid such inhibitory effects.

A range of lysozyme dosages from 0.45 to 22.5 kg/m^3 was next investigated, using activated sludge (data not shown). INTF yields with methanol were increased by up to 30%. The maximum effect was observed at a dosage of 6.75 kg/m^3, which is well in excess of that employed by Ryssov-Nielsen (1975). No improvement of INTF yield with DMSO was observed. The experiment was repeated with a smaller range of dosages (Fig. 4-2). Again, INTF yields with methanol were increased substantially (by up to 41% at 5 kg/m^3 lysozyme). INTF yields with DMSO, which remained approximately constant between 0 and 10 kg/m^3 lysozyme, were well above the maximum yield obtained with methanol. This result is probably due to the inability of lysozyme to permeabilize all the

Table 4-5. Effect of lysozyme on INTF yields from activated sludge and filamentous bacteria[a]

Sample	INTF, μM	
	Control	Lysozyme
Act. sludge, Main St.	11.6 ± 1.3	11.1 ± 1.1
Act. sludge, UF	26.3 ± 1.1	26.9 ± 1.3
Type 1701	51.3 ± 4.5	9.5 ± 1.6
S. natans	16.6 ± 1.6	14.2 ± 1.4

[a]0.07 kg/m^3 lysozyme was added at the beginning of the incubation period; INTF was extracted with DMSO

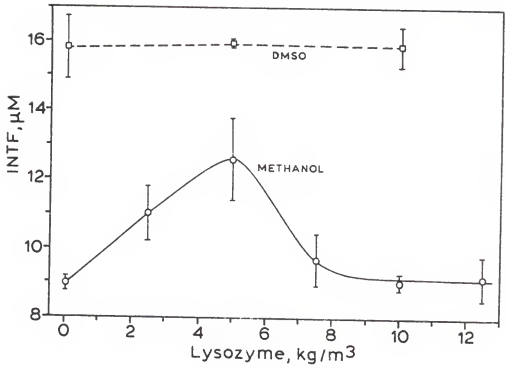


Figure 4-2. Effect of lysozyme on INT-formazan yields from activated sludge

active cells in the activated sludge. The cell walls of some gram-positive bacteria are insensitive to lysozyme, whereas gram-negative bacteria have an outer membrane which limits penetration of the enzyme (Schnaitman, 1981).

4.3.1.5. Effect of Triton X-100

The nonionic detergent Triton X-100 (iso-octylphenoxypolyethoxyethanol) is well known for its permeabilizing effect on the cytoplasmic membrane (Kagawa, 1972; Helenius and Simons 1975, Miozzari et al., 1978). Another of its properties is the ability to solubilize INTF in aqueous reaction mixtures, which is the basis for its utilization in the in vitro dehydrogenase assay. Triton X-100 shifts the site of INT reduction in the electron transport system to the flavoproteins (Zimmerman, 1975) and has been reported to enhance in vitro INT-dehydrogenase activity at concentrations of 0.16-0.46% (v/v) (Owens and King, 1975; Jones and Simon, 1979).

The effect of incorporating 0.9% (v/v) Triton X-100 into the reaction mixture at the beginning of the incubation period is shown in Table 4-6. As with lysozyme, yields from activated sludge were unaffected whereas the yield from filamentous bacteria (S. natans) was substantially reduced. In the following experiment, the effect of a range of Triton X-100 dosages on INTF yields from activated sludge was tested. The surfactant was added at the end of the incubation period in order to avoid inhibition of INT reduction. Triton X-100 substantially improved INTF yields

Table 4-6. Effect of Triton X-100 on INTF yields from activated sludge and S. natans[a]

Sample	INTF, μM	
	Control	Triton X-100
Act. sludge, Main St.	34.9 ± 2.9	35.5 ± 5.4
Act. sludge, UF	26.6 ± 1.9	25.4 ± 2.6
S. natans	14.6 ± 1.5	1.0 ± 0.1

[a]0.9% Triton X-100 was added at the beginning of the incubation period; INTF was extracted with DMSO

with methanol (by up to 56% at 0.4% surfactant) whereas there was no improvement of the yield obtained with DMSO (Fig. 4-3). At the optimum Triton X-100 dosage, the yield with methanol (20.8 $\pm$ 0.36 uM) was equivalent to that obtained with DMSO (19.9 $\pm$ 0.42 uM). Extractions on the reaction mixture amended with 0.4% surfactant were subsequently carried out with TA. The INTF yield with TA, 20.9 $\pm$ 0.44 uM, was nearly identical to that obtained with methanol.

INTF yields with DMSO, as well as with methanol, declined at the greater Triton X-100 dosages. This was attributed to leakage of formazan from microbial cells into aqueous supernatant during the dewatering (centrifugation) step of dehydrogenase assay. Cell leakage was indicated by the appearance of a reddish tint in the supernatant.

4.3.2. Correction for Abiotic INT Reduction

Abiotic reduction may be assessed by carrying autoclaved samples through the assay procedure to serve as blanks (Lopez et al., 1986). Autoclaving changes the basic nature of the sample, however, causing lysis of cells and concomitant release of intracellular, biochemical reductants. Furthermore, chemical reductants originally present in the sample can be oxidized or lost through volatilization. An alternative technique employed by Klapwijk et al. (1974) was to carry an extra set of tubes through the dehydrogenase assay: one set was incubated for 5 min and the other set for t + 5 min, where t was the desired length of the

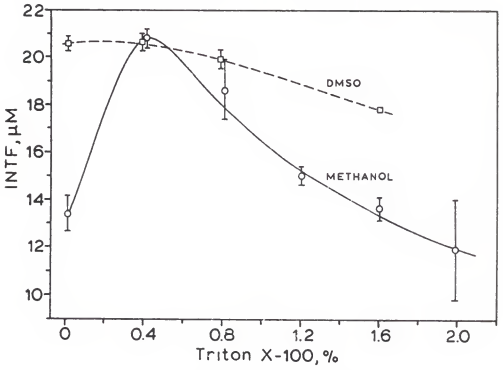


Figure 4-3. Effect of Triton X-100 on INT-formazan yields from activated sludge

reaction period.

Shown in Figure 4-4 are relationships between uncorrected O.D. and incubation time for control (top) and ferrous sulfate treated (bottom) samples. INT reduction was most rapid within the first 5 min. Thereafter, an approximately constant rate was observed. The more rapid, initial rate may be partly due to chemical reduction. Even in the absence of chemical reductants this form of relationship may be expected, however, because INT reduction may become slower as cells accumulate INTF crystals. The O.D. of the blanks (0.074 and 0.132, respectively) was time invarient. After applying the blank corrections, the curves of O.D. versus time still exhibited an initially rapid period of reduction (Fig. 4-5, top). DHA values calculated on the basis of corrected O.D. after 20 min of incubation were 41.5 and 25.0 mg O_2*/g VSS d, respectively.

The data from Figure 4-4 were replotted according to the technique of Klapwijk et al. (1974) at the bottom of Figure 4-5. Because the initial 5 min period of INT reduction is subtracted from each subsequent O.D. reading, the initial, nonlinear portion of the reaction is eliminated and the data are well described by linear relationships. The DHA values calculated on the basis of 20 min reaction time were 24.5 mg O_2*/g VSS d and 24.0 mg O_2*/g VSS d for the control and ferrous sulfate treated sample, respectively. These values are somewhat smaller than those calculated using the blank correction. However, if the slope of

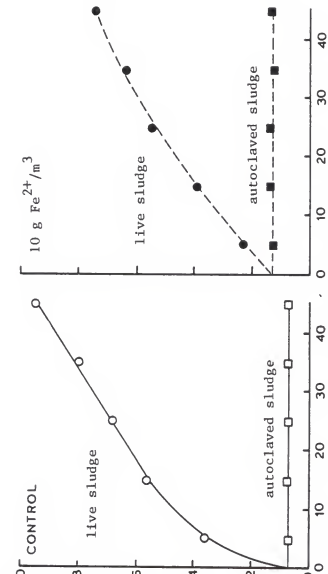


Figure 4-4. INT reduction by live and autoclaved sludge samples in the absence and presence of ferrous sulfate

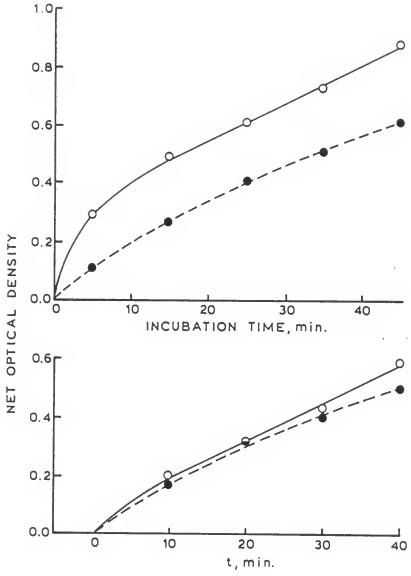


Figure 4-5. Comparision of blank correction techniques. Top; blank = autoclaved sample, Bottom = live sample incubated for 5 min

the relationships at the top of Figure 4-5 is used to calculate DHA, values of 19.9 and 20.4 mg O_2*/g VSS -d are obtained, which are very close to those calculated according to the method of Klapwijk et al. (1974).

The INT test appears to be most sensitive to ferrous sulfate during the initial few minutes of incubation. From Figure 4-5 it is seen that the instantaneous rate of INT reduction by the control fell to about the same that of the iron treated samples after only 5 min. The data plotted according the Klapwijk method (Fig. 4-5, bottom) show no difference in INT reduction between the control and iron treated samples. Thus, the cancellation of data from the initial period of INT reduction by the Klapwijk method is a major disadvantage of this technique.

4.3.3. Termination of INT Reduction and Preservation of INTF

The effect of alternative quenching solutions on the relative INTF yield of INT treated samples stored for 7 and 14 days is shown in Table 4-7. Activated sludge dosed with various concentrations of ferrous sulfate was used as the sample source. Formazan yields decreased with time, indicating that the treatments were effective in stopping INT reduction. Increases in formazan yield with time would be expected if INT reduction had continued. After one week, samples treated with 2.4N HCl had INTF yields closest to the initial (2.5 hour) value. After two weeks, the relative yields were approximately the same for all treatments. Interestingly, the two HCl treatments differed in

Table 4-7. Effect of quench solutiuon on formazan yield of INT treated samples of activated sludge after prolonged storage

Storage period	Optical density of extract, % of value at 2.5 hours			
	Quench solution			
day	HCHO	12N HCl	$HCHO/H_3PO_4$	2.4N HCl
7	73.2 ± 8.0	89.3[a] ± 8.4	77.2 ± 7.0	95.6[b] ± 11.5
14[c]	78.6 ± 9.7	74.3 ± 3.3	75.7 ± 8.6	79.5 ± 6.9

[a]Significantly greater than HCHO treatment at $p < 0.01$
[b]Significantly greater than HCHO and $HCHO/H_3PO_4$ treatments at $p < 0.01$
[c]Means not significantly different from each other at $p < 0.01$

effectiveness despite their equal final concentrations. This result may derive from better initial mixing achieved with the more dilute acid.

INTF yields of sample treated with 2.4N HCl were measured between 0 and 8 days storage. Formazan yields decreased with time during 8 days storage as shown in Figure 4-6. Changes in the pH of activated sludge samples treated with alternative quenching solutions were observed. Samples were stable in terms of pH variation during three weeks prolonged storge and the average pH values were 6.46, 0.88, 1.46 and 1.04 with quenching solution of HCHO, 12N HCL, HCHO/H_2PO_4 and 2.4N HCl repectively (Figure 4-7).

The effect of quench solutions on the yield of INTF from INT treated S. natans samples after prolonged storage is shown in Table 4-8. The 12N HCl, formaldehyde and formaldehyde/H_3PO_4 allowed INT reduction to continue after treatment, as indicated by 7-day INTF yields which were 56-67% greater than the 3-hour yields. Treatment with 2.4N HCl limited further INT reduction to 21% after 7 days. INTF yields of the formaldehyde and formaldehyde/$H3PO_4$ treated samples decreased with time when the storage period exceeded 7 days, whereas the yield of the 12N HCl treated samples continued to increase with extended storage. Yields of the 2.4N HCl treated samples were nearly invarient with time.

The preserved samples of activated sludge and S. natans were examined using bright field microscopy. In sludge samples which

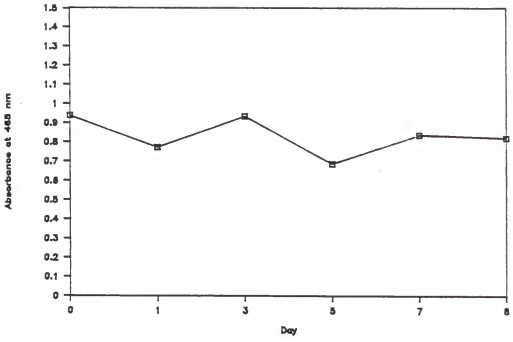


Figure 4-6. Variation in INTF of samples treated with 2.4N HCl between 0 and 8 days

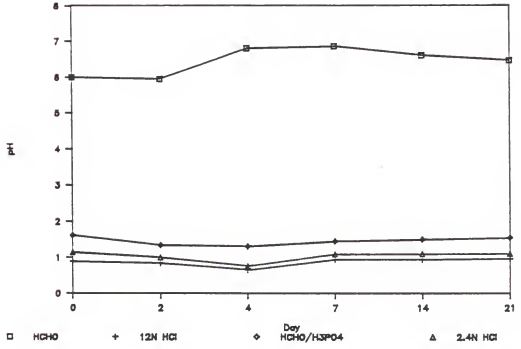


Figure 4-7. Variation in pH of samples treated with alternative quenching solutions

Table 4-8. Effect of quench solution on fromazan yield of INT treated S. natans samples after prolonged storage

Storage period,	Optical density of extract, % of value after 3 hours			
	Quench solution			
	HCHO	12N HCl	$HCHO/H_3PO_4$	2.4N HCl
7	165	167	156	121
14	146	191	131	123
21	119	200	91	127

had been dosed with ferrous sulfate, the formaldehyde or 12N HCl treatments caused formazan crystals to disperse after 2-3 weeks. Formazan crystals in formaldehyde/H_3PO_4 treated samples turned blackish after 3 weeks. The most stable formazan crystals were observed in 2.4N HCl treated samples.

CHAPTER 5
ASSESSMENT OF FERROUS SULFATE TOXICITY TO FILAMENTOUS BACTERIA AND ACTIVATED SLUDGE

5.1. Introduction

The INT-dehydrogenase test developed by Bitton and Koopman (1982) makes it possible to differentiate between the activity of filamentous biomass and gross (floc former plus filamentous) biomass in activated sludge. The test relies on microscopic observation of active (containing red formazan crystals) and non-active (red crystals absent) lengths of extended filaments in INT treated sludge samples to assess filamentous dehydrogenase activity (DHA_c). The test also provides for determination of gross dehydrogenase activity (DHA_e) in the same sludge sample. This is accomplished by extraction and colorimetric measurement of the INTF formed during the incubation.

Most bacterial species tested can reduce INT (Quinn, 1984), but a few exceptions have been noted. For example, Buchanan-Mappin et al. (1986) failed to observe formazan crystal development by bacteria in a mountain stream and in groundwater. The majority of bacteria isolated from the stream water were identified as Pseudomonas spp. However, Dutton et al. (1983) found INT reduction and characteristic INTF crystal formation by Pseudomonas fluorescens. In order to determine DHA_c, bacterial

filaments must reduce INT and form resoluable INTF crystals. Furthermore, it is desirable that the counting technique yield an activity value that correlates with other measurements of activity such as ATP and oxygen uptake rate.

It is now known that about 25 different filamentous microorganisms can grow in activated sludge and cause bulking (Farquar and Boyle, 1971a,b; Eikelboom, 1975, 1977; Eikelboom and van Buijsen, 1981; Strom and Jenkins, 1984). We picked as representative filamentous bacteria type 021N, Thiothrix and S. natans, which are the 3rd, 4th and 6th most common filaments in bulking sludges found in USA plants (Blackbeard et al., 1986). These bacteria were available in pure cultures originally isolated from activated sludge.

The objective of the work reported in this chapter was to establish the validity of the INT-dehydrogenase test for assessing toxic inhibition of filamentous bacteria and activated sludge by ferrous sulfate. Oxygen uptake rate and ATP were used as comparative parameters because of their widespread acceptance for determining sludge activity.

5.2. Materials and Methods

5.2.1. Cultures and Growth Media

Three types of filamentous microorganisms (S. natans isolate FA3, type 021N isolate N2 and Thiothrix strain A1) were grown in the laboratory. Lyophilized cultures of S. natans and type 021N were provided by Dr. Michael Richard (Colorado State University,

Fort Collins, CO). Seed cultures of Thiothrix on LTH agar slant were provided by Dr. Terry Williams (Pennsylvania State University, University Park, PN). The lyophilized cultures of S. natans and type 021N were reactivated using CGY broth (Dondero et al., 1961) as follows.

1. Puncture the vial tip to release the vacuum with a red-hot needle over a flame.
2. Break off vial tip at scoring.
3. Swab inner vial with alcohol and aseptically remove inner cotton plug.
4. Aseptically pipette (pasteur pipette) 0.5 mL CGY broth into the vial and withdraw contents; add this to the CGY broth (100 mL per 250 mL flask) and incubate at 24°C.
5. Plate on CGY agar and select a typical colony for transfer to CGY slants (containing 1 mL sterile water), store in the refrigerator and subculture every 3 weeks because autolysis occurs rapidly.

S. natans was grown in GMB medium (Lau et al., 1984), type 021N in GMBN (Richard et al., 1984) and Thiothrix in LTH (Williams and Unz, 1985) for the toxicity tests. These media were recommended by the investigators supplying the pure cultures and gave satisfactory growth of the bacteria in the form of filaments, as desired. (It is possible for many of the filamentous bacterial types to grow as single cells under certain conditions, e.g., in rich media). Media recipes are given in Table 5-1. For GMB and

Table 5-1. Culture media recipes

Constituent	Final concentration, kg/m^3			
	CGY[a]	GMB[b]	GMBN[c]	LTH[d]
casitone	5.0			
glycerol	10.0			
yeast extract (BBL)	1.0			
agar (Difco, Bacto-Agar)	13.0[e]			
glucose		1.0	1.0	
$(NH_4)_2SO_4$				0.50
NH_4Cl		0.36	0.36	
$MgSO_4.7H_2O$		0.10	0.15	0.10
$CaCl_2$		0.02	0.20	0.05
$FeCl_3.6H_2O$		0.003	0.003	0.002
EDTA-Na		0.003	0.003	0.003
KH_2PO_4		0.30	0.08	0.085
K_2HPO_4		1.80	0.11	0.11
$NaHCO_3$			0.42	
Sodium lactate				1.0
Sodium thiosulfate				0.5
vitamin mix		1.0 mL	1.0 mL	1.0 mL

a Dondero et al. (1961)
b Lau et al. (1984)
c Richard et al. (1985)
d Williams and Unz (1985)
e Agar was used for slants.

GMBN media all stock solutions except the vitamin mixture were made up at 20x final concentration. $FeCl_3.6H_2O$ and EDTA-Na were combined in the same solution, as were KH_2PO_4 and K_2HPO_4. All other components were made up separately. The stock solutions were autoclaved individually and 50 mL of each solution was added asceptically to autoclaved, deionized water to make one litre of medium. Among the major salt solutions, $CaCl_2$ was added last to avoid precipitation. The vitamin mixture (Table 5-2) was made up at 1000x final concentration and was sterilized by passage through a 0.22 μm membrane filter. LTH broth was prepared in the same manner except for the use of autoclaved HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; 0.01 M, pH 7.6) (Sigma) instead of autoclaved, deionized water as used in GMB and GMBN. One mL of the vitamin mixture was added asceptically to 1 litre of medium. All media were adjusted to pH 7.6 with 1 N NaOH.

Eikelboom's key (1975, 1977) as modified by Strom and Jenkins (1984) was used to identify filamentous microorganisms to type. The microscope was a Nikon Optiphot with a 35 mm camera attachment and autoexposure accessory. The gram stain, modified Neisser's stain, intracellular sulfur granule test, and sheath stain (Richard et al., 1981) were used in the identification procedure. (These staining procedures are described in Appendix A). Filamentous microorganism identifications were confirmed by Dr. Richard at Colorado State University.

Cultures were grown in 2.0 L, narrow mouth Erlenmeyer flasks

Table 5-2. Vitamin mixture used in GMB, GMBN and LTH growth media (After Eikelboom, 1975)

Constituent	Final concentration, g/m^3
Ca pantothenata	0.1
niacin	0.1
biotin	0.005
cyanocobalamin	0.005
folic acid	0.005
pyridoxine	0.1
p-aminobenzoic acid	0.1
cocarboxylase	0.1
inositol	0.1
thiamine	0.1
riboflavin	0.1

(1.0 L liquid volume). Flasks were stoppered with cotton plugs and oscillated on a gyrotary shaker (100 cycles/min) to provide aeration. Toxicity tests were conducted after 5-7 days of incubation.

Activated sludge was obtained from a completely mixed process treating domestic and commerical wastewaters from the city of Gainesville's Main Street plant. The mean organic loading at this facility was 0.10 grams five-day biochemical oxygen demand daily per gram volatile suspended solids under aeration.

5.2.2. Experimental Procedures

5.2.2.1. Growth of S. natans in CGY Medium

Lyophillized culture of S. natans was activated in the CGY broth, plated on CGY agar and observed by phase contrast microscopy. The activated culture was inoculated into 500 mL CGY broth in a 1 L Erlenmeyer flask. The cultures were grown on a gyrotary shaker (Lab Line model 3590; 100 oscillations/min) at 27-28°C. The turbidity of 50 mL subsamples was measured by a Hach model 2100A turbidity meter.

5.2.2.2. Effect of pH on $FeSO_4$ toxicity to S. natans

S. natans was grown in CGY medium and pH was adjusted from 5.0 to 8.5 by adding 0.1N HCl or 0.1N NaOH to the medium. The experiment was conducted after reaching the declining growth phase (6 days after inoculation). The dry weight of test culture was 354 g/m^3. 20 g/m^3 of ferrous sulfate (as Fe) was added to 100 mL aliquots of the culture in 250-mL Erlenmyer flasks. Treated

aliquots at different pH values were agitated for two hours at 100 oscillations/min. pH was adjusted to 7.6 by adding 0.1N HCl or 0.1N NaOH after the two hour contact time before assaying for gross dehydrogenase activity (DHA_e). Duplicate 5 mL subsamples from each aliquot were assayed.

5.2.2.3. Iron inhibition of filamentous bacteria

Toxicity tests were conducted after 5-7 days cultivation of pure cultures in the respective media. Prior to each experiment, the cultures were harvested by centrifuging for 15 min at 1,500xg. The supernatant was discarded and the settled biomass was resuspended in sterile deionized water at pH 7.6. The dry weight of culture was adjusted to 300-400 g/m^3.

Known amounts of ferrous sulfate were added to 100 mL aliquots, which were agitated for 2 hours at 100 oscillations/min. The pH was adjusted to 7.6 using 0.1N NaOH before assaying the activity of pure cultures. Four activity parameters (DHA_e, DHA_c, ATP, and OUR) were determined in parallel on split aliquots of the cell suspensions. Duplicate measurements were made for each parameter except OUR.

5.2.2.4. Iron inhibition of activated sludge

10 litres of activated sludge and 10 litres of unchlorinated, secondary effluent from Gainesville's Main Street plant were obtained. The sludge was aerated continuously once it was in the laboratory. Diluted sludge suspensions were formed by combining 500 mL activated sludge with 500 mL secondary effluent. The

volatile supended solids concentration of the diluted sludge suspensions was 981 g/m^3. The appropriate volume of ferrous sulfate (stock solution strength = 1 kg Fe^{2+}/m^3) was added to the secondary effluent before combining the two volumes. A contact time of 2 hours with agitation at 100 oscillations/min was allowed. The pH was adjusted to 7.6 using 0.1N NaOH before assaying for sludge activity. Three activity parameters (DHA_e, ATP and OUR) were measured in parallel on split aliquots of the sludge suspensions. Duplicate subsamples from each treatment were assayed for each activity parameter except OUR.

5.2.3. Analytical Methods

5.2.3.1. INT-dehydrogenase activity

INT solution (3.95 mM) was prepared by adding 200 mg of INT (Eastman Kodak) to 100 mL of distilled water. The pH of distilled water was adjusted to pH 7.6 to promote dissolution. The INT solution was filtered through 0.22 μm filters to sterilize the solution. INT solution was stored in foil wrapped, glass bottles at 4°C for a maximum of three weeks. INT was added in the ratio of 0.5 mL INT per 5.0 mL of sample, giving an initial in situ concentration of 0.36 mM. Incubation with INT was at 22 $\pm$ 2°C in the dark with agitation on a shaker table (Model 3590, Lab line) at 100 oscillations/min. The reaction was stopped after 20 min by adding 1.0 mL of 2.4 N HCl per 5.0 mL of original sample. 6.5 mL of INT-treated and preserved mixture was centrifuged at 1,500x g for 10 min, after which the supernatant was poured off.

Five mL of DMSO was added and the tube was capped. The pellet was resuspended by shaking for 15 seconds and the INTF was extracted 15 min in darkness at 22 ± 2°C. The extract was clarified by centrifuging at 1500x g for 10 min. Absorbance of the supernatant was read at 465 nm using a 1 cm path length. INT-dehydrogenase activity was calculated in equivalent oxygen uptake (O_2*) units using the following equation:

$$DHA_e = \frac{1002\ D_{465}\ v}{V\ C\ t\ F} \qquad (5\text{-}1)$$

where D_{465} = optical density at 465 nm through a path length of 1 cm, v = final solvent volume (mL), V = volume of INT-treated sample used in the extraction procedure (mL), C = initial biomass concentration of the sample (kg VSS/m^3), t = incubation time (min) and F = factor to adjust for dilution caused by adding INT and HCl = 0.769. Equation (5-1) was derived from the basic expressions presented by Lopez et al. (1986) using the extinction coefficient of 23 000 $M^{-1}cm^{-1}$ for DMSO that was reported in Chapter 4.

DHA_c was determined by microscopic observation of prepared specimens (Bitton and Koopman, 1982). When counting S. natans, smears were made of INT-treated mixture and allowed to air dry. The dry smears were covered with 0.05 % malachite green (Allied Chemicals) for 1 min. Malachite green was drained completely off the slides and the smears allowed to air dry prior to examination

under oil emulsion at 1000 x using bright field microscopy. When counting type 021N and Thiothrix, wet smears were examined without counterstaining at 400x or 640x magnification using bright field microscopy. It was necessary to count smears made of Thiothrix culture immediately after adding immersion oil, as the oil slowly dissolved the formazan crystals. Dissolution of formazan crystals in S. natans or type 021N was not a problem. Counts were made of active (red INTF crystals present) and inactive (INTF crystals absent) lengths of filaments. Six to seven fields were selected randomly on each of 3 slides and approximately 30 counts of 0.7 μm long portions of filament were enumerated in each field. DHA_c was calculated according to:

$$DHA_c, \% = \frac{\text{Length of active filaments}}{\text{Total filament length}} \times 100 \qquad (5\text{-}2)$$

5.2.3.2. Adenosine triphosphate

ATP was determined by a modified version of the method developed by Patterson et al. (1970). 50 mg firefly-lantern extract (FLE 50, Sigma), which was stored at -20 °C before use, was reconstituted one day prior to the experiment by adding 5 mL deionized water and shaking vigorously for 30 seconds. It was stored at 4°C until use. Reaeration in later steps was avoided. Reconstituted firefly-lantern extract was kept in an ice bath during ATP measurement. 7 mL of 0.025 M tris buffer (pH = 7.6) and 1 mL of 0.048 M EDTA-Na prepared in 0.025 M tris buffer were placed in a sand bath which was uniformly heated to induce a mild

boiling action in the test tube. 2 mL of sample was injected into boiling tris and ATP was extracted for 3 min. The extract was cooled rapidly in an ice bath and the final volume was adjusted to 10 mL with 0.025 M tris buffer. Extracts were stored at -20°C for up to 3 weeks. After thawing, the solution was centrifuged for 10 min at 1500 x g. 0.1 mL of the supernatant was added to a reaction cuvette and the cuvette was inserted into reaction chamber of an ATP photometer (J4-7441, American Instrument Co.). 0.2 mL reconstituted firefly-lantern extract solution was injected into the reaction chamber using a sterile needle. Light emission was recorded for 10 sec as accumulated voltage (J4-7426A, American Instrument Co.). ATP concentration was determined from a standard curve. ATP stock solution (100 g/m^3) was prepared by dissolving crystalline ATP (Sigma) in tris buffer and was stored at -20°C until needed. Standards (usually 0.01-0.2 μg ATP/mL) were made by futher dilution of stock solution with Tris. A typical ATP standard curve is shown in Fig. 5-1.

5.2.3.3. Oxygen uptake rate

A 50 mL Erlenmeyer flask containing a magnetic stirring bar was filled completely with pH-adjusted medium and was placed on a magnetic stirrer. A galvanic oxygen electrode (New Brunswick Scientific) was inserted into the flask and the top of the container was sealed with plastic film to prevent oxygen transfer from the atmosphere. DO was monitored and recorded with a DO

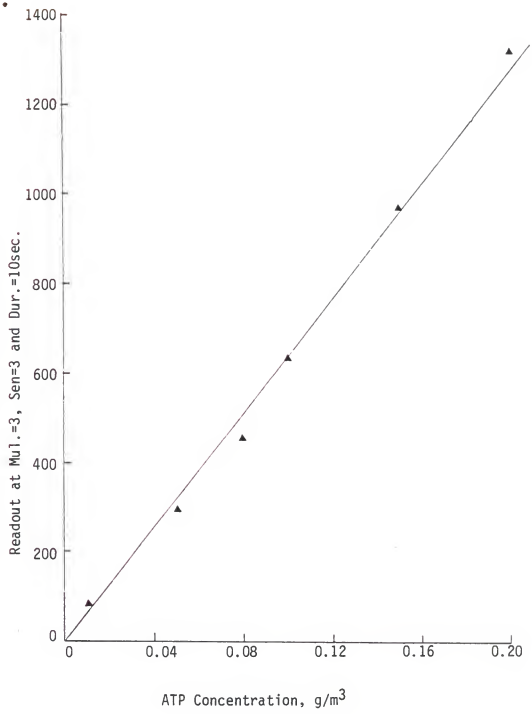


Figure 5-1. Typical ATP standard curve

analyzer and recorder (DO 40, New Brunswick Scientific; Rustrak 288, Gulton) as a function of time for 20 min or until DO reached 1.0 g/m^3. A slope was determined from the recording using only the linear portion of the readout. OUR was calculated from the following equation:

$$OUR = 1.44 \frac{S}{C} . \qquad (5-3)$$

where OUR = oxygen uptake rate (mg O_2/g VSS-d), S = slope of the linear portion of the DO versus time recording (mg O_2/L-min), and C = biomass concentration of sample (kg VSS/m^3).

5.2.3.4. Adsorbed and free iron

10 mL iron treated sample was centrifuged at 1,500x g for 20 min. The supernatant was poured into a 50 mL flastic bottle and the pH lowered by adding 0.5 mL of concentrated HCl. 10 mL concentrated HCl was added to the pellet. Twelve hours were allowed for iron dissolution to occur. The two fractions were diluted to a total volume of 100 mL each with deionized water, then filtered through 0.45 μm pore size membrane filter paper (Gelman GN-6) to remove particulates. Iron was measured using atomic absorption spectrophotometry (Perkin Elmer model 500). All instrument settings followed the manufactuer's recommendations for standard operation conditions (air-acetylene flame, wavelength; 248.3 nm, slit; 0.2 nm, hollow cathode lamp current; 0.2 ma, integration time; 1 sec, and lamp energy; 54).

5.2.3.5. Turbidity and pH

The growth phase of the culture was checked by the measurement of turbidity (Hach, model 2100A). The standard tubes (cat. no. 2480 and 2481) were used to standardize the instrument. pH was measured by an electrode analyzer system (601A, Orion). The electrode was calibrated by buffer solutions (pH = 7 and 10) before use.

5.3. Results and Disscussion

5.3.1. Growth of S. natans in CGY Medium

Turbidity increased exponentially for 5 days after inoculation as shown in Fig. 5-2. After 6-7 days, the cells entered the declining growth phase.

5.3.2. Effect of pH on Toxicity of Ferrous Sulfate to S. natans

The dehydrogenase activity of S. natans treated with 20 g Fe^{2+}/m^3 was substantially affected by pH. As shown in Fig. 5-3, the activity at pH 7.5 - 8.0 was more than 10 times that at pH 5.0. In comparison, the activity of the control at pH 7.6 was 287 mg O_2/g VSS-d.

This behavior may be due in part to the effect of pH on the availability of ferrous ion, which is the more inhibitory form. As shown by Stumm and Lee (1961), the rate of oxidation is proportional to the square of the hydroxide ion concentration at pH values in excess of 8.5. Under aerobic condition and neutral pH, $Fe(OH)_3$ is the dominant form. When ferrous iron salts are added to water under aerobic conditions, ferrous iron is oxidized

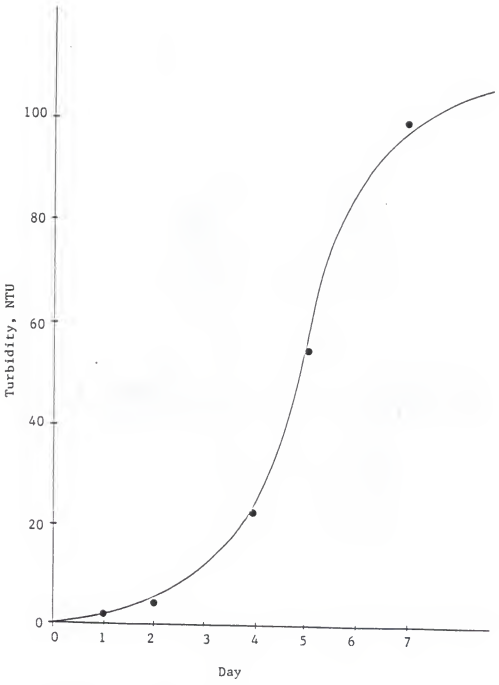


Figure 5-2. A growth curve of Sphaerotilus natans in CGY broth

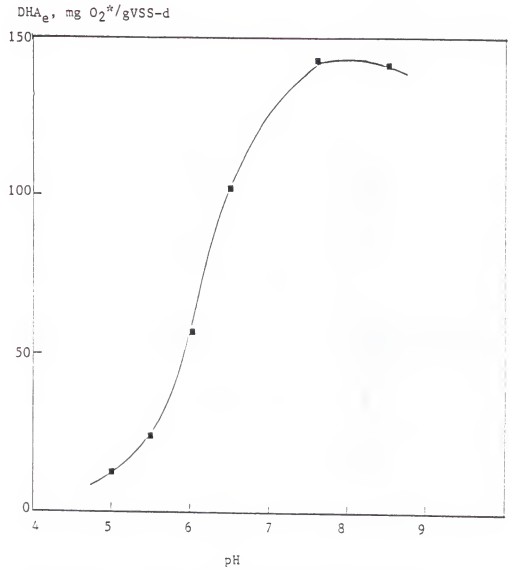


Figure 5-3. Effect of pH on dehydrogenase activity of S. natans treated with 20 g/m^3 ferrous sulfate (as Fe)

to ferric iron by oxygen. Ferric iron then precipitates readily as ferric hydroxide. Because the solubility of the ferrous precipitate (ferrous carbonate) is higher than that of ferric hydroxide.

5.3.3. Assessment of Iron(II) Toxicity to S. natans

The culture of S. natans exhibited typical batch growth charateristics of S. natans, i.e., single cells or filaments of $\leq$ 10 cells in length, very frequent sheath formation, and growth in large flocs. Data for all toxic inhibition trials is given in Table 5-3. Correlations and their statistics are given in Table 5-4. The ferrous sulfate concentration which inhibits 50% of filamentous bacteria in pure culture and activated sludge (IC_{50}) was determined as follows. The degree of inhibition at each Fe^{2+} dose was determined by comparing the activity values of each parameter to the control of each parameter. The degree of inhibition was transformed according to the following equation (Johnson et al., 1974):

$$\Gamma = \frac{\text{\% inhibition}}{100 - \text{\% inhibition}} \qquad (5\text{-}4)$$

A plot of Γ versus dosage approximated a linear relationship. Lines of best fit to the transformed data were determined by least squares linear regression. The IC_{50} was taken as the dosage at which the best fit relationship gave a value of Γ equal to 1. IC_{50}s determined by DHA_e, DHA_c, ATP and OUR were 34.8, 20.7, 48.1 and 28.7 mg Fe/g VSS, respectively (Fig. 5-4). As shown in Fig.

Table 5-3. Toxicity data for filamentous bacteria and activated sludge

Cultures	Fe dosage mg Fe/g VSS	Gamma* DHA_e	DHA_c	ATP	OUR
S. natans	6.39	0.054	0.094	0.054	0.114
	12.78	0.645	0.656	0.192	0.597
	19.17	0.788	0.835	0.346	0.767
	31.95	0.908	1.410	0.603	0.862
	47.92	1.330	2.803	0.927	1.273
	63.90	1.630	4.293	1.420	2.774
Type 021N	2.66	0.362	0.362	0.533	0.231
	5.32	0.490	0.497	0.681	0.434
	7.98	0.653	0.515	0.742	1.156
	10.64	0.728	0.981	0.818	1.332
	13.30	0.869	0.927	1.364	1.596
	26.60	1.057	1.770	2.876	2.533
	39.90	2.520	3.856	3.694	---
Thiothrix	1.25	0.701	0.312	0.093	0.337
	2.50	1.525	1.151	0.513	0.845
	5.00	1.994	2.311	0.435	1.724
	7.50	2.247	2.155	1.020	2.059
	10.00	3.082	2.236	1.688	2.389
	15.00	5.849	5.289	2.236	3.855
	25.00	13.706	16.857	2.460	4.650
Activated sludge	5.10	0.040		0.142	0.078
	10.20	0.178		0.264	0.377
	20.40	0.479		0.337	0.637
	25.50	0.631		0.391	0.855
	30.60	0.739		0.433	0.957
	40.80	1.070		0.927	1.203
	51.00	1.558		1.415	2.236

*$\text{Gamma} = \frac{\text{\% inhibition}}{100 - \text{\% inhibition}}$

Table 5-4. Correlations between the DHA_e and alternative activity parameters (DHA_c, ATP, and OUR)*

Cultures	Parameters compared	Line of best fit	r^2
S. natans	DHA_e, DHA_c	Y = 1.390X + 21.761	0.968
	DHA_e, ATP	Y = 210.213X - 13.029	0.867
	DHA_e, OUR	Y = 0.525X + 5.897	0.964
Type 021N	DHA_e, DHA_c	Y = 2.061X + 16.303	0.976
	DHA_e, ATP	Y = 97.922X + 22.317	0.934
	DHA_e, OUR	Y = 0.720X + 37.025	0.912
Thiothrix	DHA_e, DHA_c	Y = 1.496X + 0.151	0.959
	DHA_e, ATP	Y = 195.148X - 19.751	0.845
	DHA_e, OUR	Y = 0.804X - 7.034	0.959
Activated sludge	DHA_e, ATP	Y = 51.619X + 6.843	0.923
	DHA_e, OUR	Y = 0.371X - 8.300	0.903

*All correlations were significant at $P < 0.01$.
Y = DHA_e, X = alternative activity parameter.

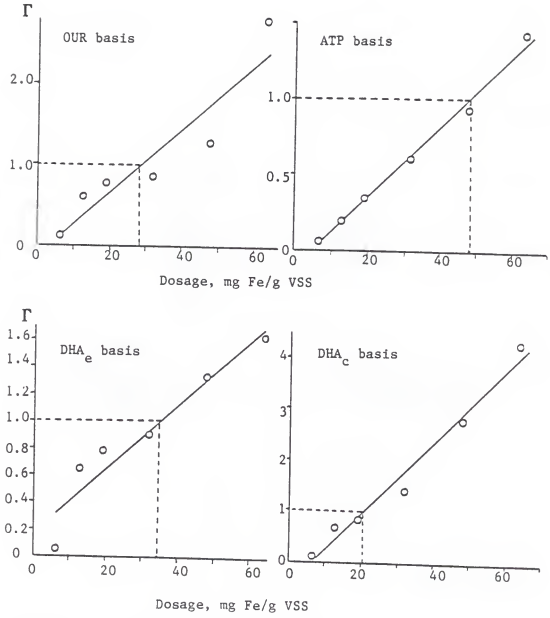


Figure 5-4. Assessment of ferrous sulfate toxicity to S. natans using oxygen uptake rate, ATP, and dehydrogenase activity parameters

5-4, linear relationships ($P \leq 0.01$) were exhibited between Γ (DHA_e, DHA_c, ATP and OUR basis) and iron dose. The coefficients of determination (r^2) based on DHA_e, DHA_c, ATP and OUR were 0.913, 0.977, 0.993, and 0.875, repectively. DHA_e was compared to OUR, ATP and DHA_c in Fig. 5-5. DHA_e was correlated linearly with DHA_c, OUR and ATP in the presence of ferrous sulfate ($P \leq 0.01$). Correlation coefficients were 0.984, 0.982, and 0.931, respectively.

5.3.4. Assessment of Iron(II) Toxicity to Type 021N

A culture of type 021N was qualified as type 021N > 99% purity. Unicellular forms observed could have been 021N gonidia. Figure 5-6 shows an illustration of decreasing INTF crystals formed as the inhibitory effect of ferrous sulfate on type 021N increases. An alternative technique in determining inhibitory effect of toxicant is demonstrated in Figure 5-7 using dark field microscopy, which is sometimes useful in detecting the position of INTF crystals formed by comparing with bright field microscopy.

IC_{50}s determined via DHA_e, DHA_c, ATP and OUR were 16.1, 12.2, 9.5 and 8.8 mg Fe/g VSS, repectively, as shown in Figure 5-8. The coefficients of determination between Γ (DHA_e, DHA_c, ATP and OUR basis) and iron dose were 0.896, 0.942, 0.976, and 0.936, repectively, at a level of significance ≤ 0.01. This result indicates that type 021N is more sensitive to ferrous sulfate than S. natans. Relationships of DHA_e to DHA_c, OUR and ATP in iron-inhibited type 021N are shown in Figure 5-9. They were linear in

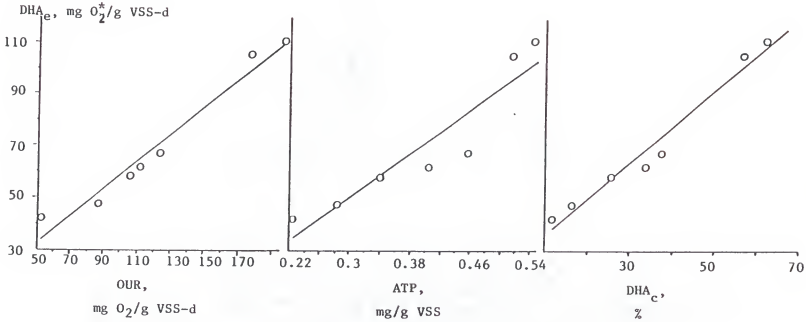


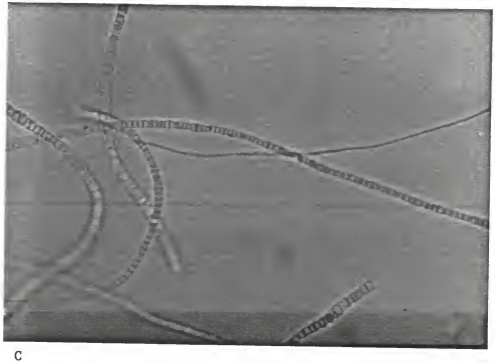
Figure 5-5. Correlation between DHA_e and alternative activity parameters in iron(II) inhibited cultures of S. natans

A

Figure 5-6. Effect of ferrous sulfate on INTF content of type 021N grown in GMBN medium during the toxicity bioassay
A - 1.0 g Fe/m^3 addition

B

Figure 5-6 (continued)
B - 5.0 g Fe/m^3 addition



C

Figure 5-6 (continued)
C - 10.0 g Fe/m^3 addition

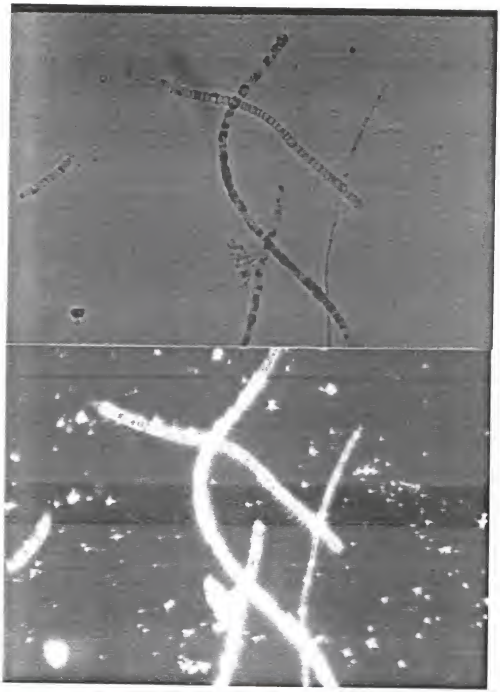


Figure 5-7. Micrographs of type 021N grown in GMBN medium
Top-640x bright field
Bottom-640x dark field

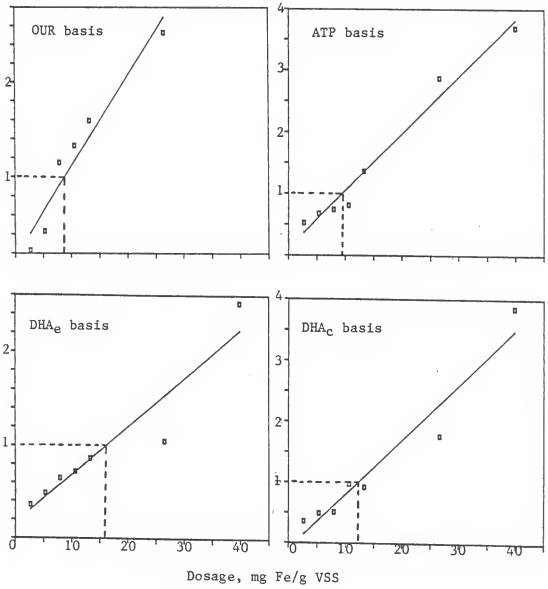


Figure 5-8. Assessment of ferrous sulfate toxicity to type 021N using oxygen uptake rate, ATP, and dehydrogenase activity parameters

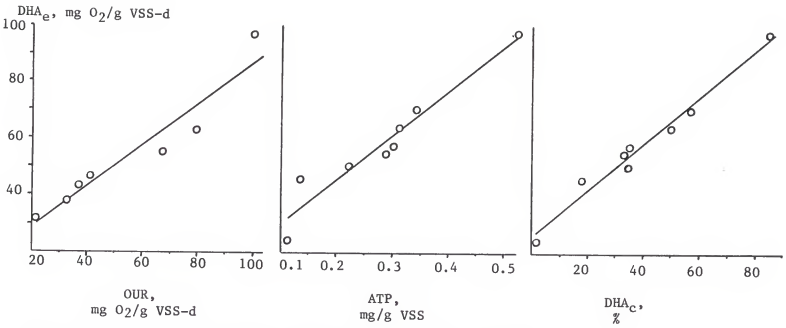


Figure 5-9. Correlation between HDA$_e$ and alternative activity parameters in iron(II) inhibited cultures of type 021N

form and highly significant ($P \leq 0.01$), and were charaterized by correlation coefficients of 0.954 or greater.

5.3.5. Assessment of Iron (II) Toxicity to Thiothrix

Growth of Thiothrix was very healthy with gonidia and rosettes apparent. Cultures of Thiothrix were also qualified for toxicity bioassay of pure cultures as shown in Figure 5-10. IC_{50}s determined via DHA_e, DHA_c, ATP and OUR were 3.5, 2.8, 7.5 and 2.5 mg Fe/g VSS, respectively, as shown in Figure 5-11. Linear correlations were found between Γ (DHA_e, DHA_c, ATP and OUR basis) and iron dose (r^2s were 0.936, 0.871, 0.869 and 0.941, repectively, $P \leq 0.01$.). These results indicate that Thiothrix sp. is the most sensitive among pure cultures of filamentous bacteria tested. DHA_e of Thiothrix in the presence of ferrous sulfate was correlated linearly with DHA_c, ATP and OUR (Fig. 5-12). The correlation coefficients were 0.979, 0.919 and 0.979, respectively, at a level of significant ≤ 0.01.

5.3.6. Distribution of Iron in Ferrous Sulfate Treated Thiothrix

As the iron concentration was increased, the iron content of Thiothrix progressively increased (Fig. 5-13). The iron concentration of the supernatant increased more slowly, eventually reaching a plateau at iron dosages above 10 g Fe^{2+}/m^3. Inhibition of Thiothrix DHA_e increased gradually with the increasing addition of iron up to 10 g Fe^{2+}/m^3, the concentration at which the inhibition reached 92%. However, as the iron addition increased over certain levels (i.e., 10 g/m^3 as Fe with the pure cultures of

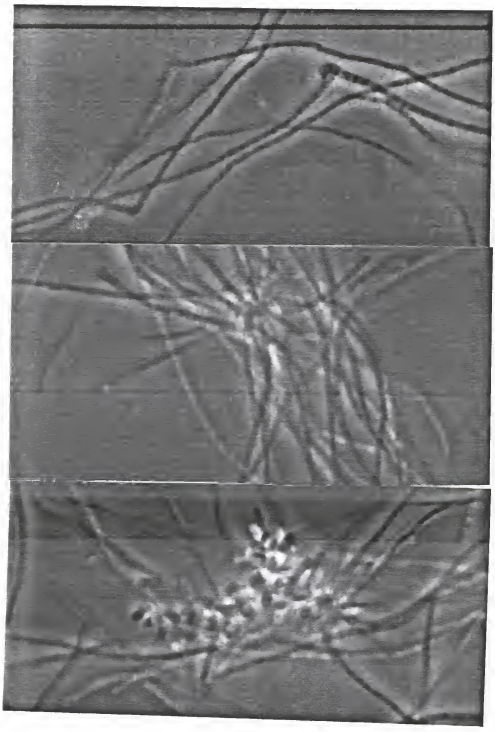


Figure 5-10. Micrographs of Thiothrix grown in LTH medium. (All micrographs taken at 1000x magnification with bright field illumination)

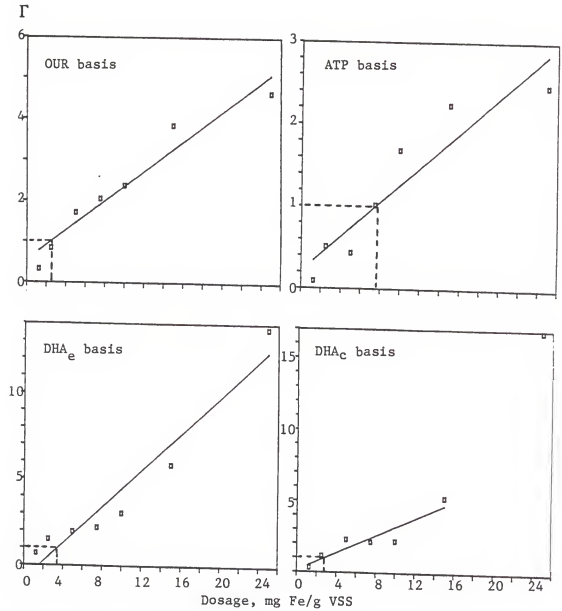


Figure 5-11. Assessment of ferrous sulfate toxicity to Thiothrix using oxygen uptake rate, ATP, and dehydrogenase activity parameters

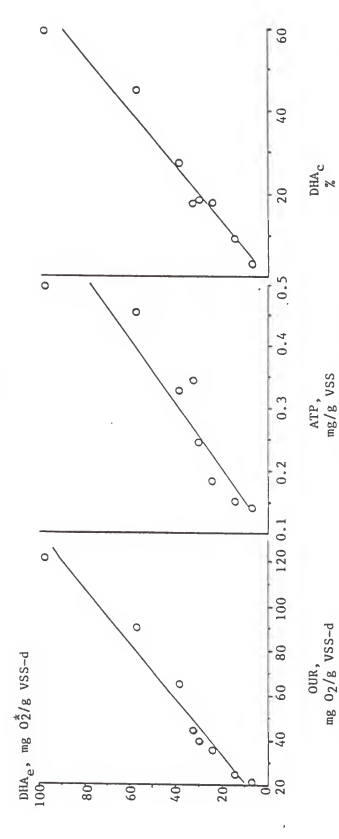


Figure 5-12. Correlation between DHA$_e$ and alternative activity parameters in iron(II) inhibited cultures of Thiothrix

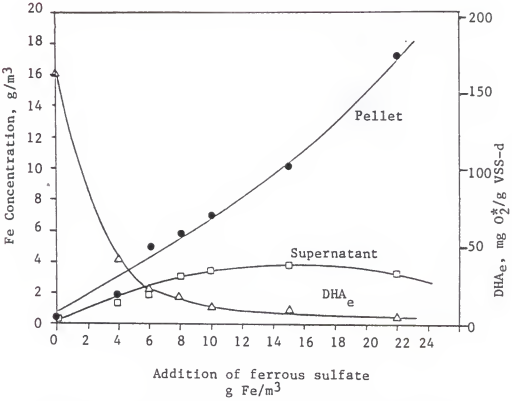


Figure 5-13. Distribution of iron in ferrous sulfate treated Thiothrix cultures (VSS = 0.155 g/L)

Thiothrix), not much inhibition was observed. The above discussion suggests that the sucess of inhibition with low doses is due to a metabolic effect of iron on the microbial populations after possibly biosorption of iron by the sheath and cell. The excessive iron sorbed may block metabolism rather than act as a barrier of nutrient uptake. The excessive amount of iron measured in the biomass of Thiothrix may exist in the forms of hydroxide or carbonate precipitates as ineffective iron.

5.3.7. Assessment of Iron(II) Toxicity to Activated Sludge

Toxicity of ferrous sulfate to activated sludge was determined via DHA_e, ATP and OUR. The IC_{50}s found were 36.6, 28.5 and 43.3 mg Fe/g VSS (Fig. 5-14). Linear relationships were observed between Γ (DHA_e, ATP and OUR basis) and iron dose resulting coefficient of determination = 0.987, 0.923 and 0.868, repectively, $P \leq 0.01$). Resistance of floc formers to ferrous sulfate on a mass dose basis was three times higher than type 021N and eight times higher than Thiothrix. Ferrous sulfate has more inhibitory effect on type 021N or Thiothrix than S. natans. or activated sludge. DHA_e correlated linearly with OUR and ATP in the presence of ferrous sulfate (Figure 5-15). The relationships had correlation coefficients of 0.950 and 0.961, respectively, at a level of significance ≤ 0.01.

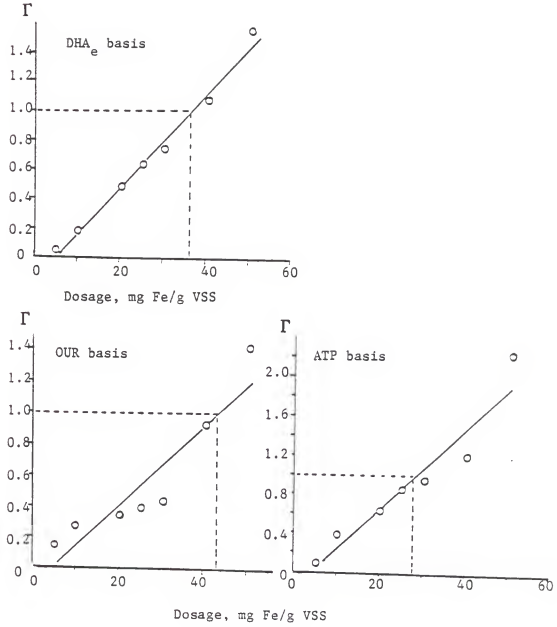


Figure 5-14. Assessment of ferrous sulfate toxicity to activated sludge using oxygen uptake rate, ATP, and DHA$_e$

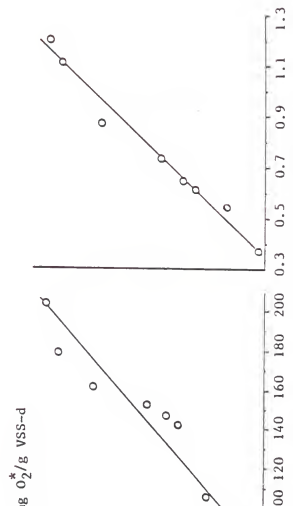


Figure 5-15. Correlation between DHA_e and alternative activity parameters in iron(II) inhibited activated sludge

CHAPTER 6
INT-DEHYDROGENASE ASSAY AS AN ADJUNCT TO BULKING CONTROL WITH FERROUS SULFATE

6.1 Introduction

It has been recognized for some time that measured addition of chlorine or hydrogen peroxide to activated sludge is an effective method for controlling sludge bulking. This effect has generally been attributed to the ability of these oxidants to kill the filamentous microorganisms extending from activated sludge flocs while leaving most of the bacteria inside the flocs uninhibited (Smith and Purdy 1936, Pipes 1974, Jenkins et al., 1982). Toxic inhibition of filaments was first quantitatively related to SVI and bulking control in activated sludge systems by Logue et al. (1983) and Koopman et al. (1984). These investigators demonstrated that the relative dehydrogenase activity of filamentous bacteria was reduced by oxidant additions. This reduction was accompanied by reductions in SVI. It was also shown that INT-dehydrogenase assay could be used to predict the proper dosage of oxidant required to control sludge bulking.

The purpose of the present study was to demonstrate that INT-dehydrogenase assay can be used as an adjunct to sludge bulking control with the reductant ferrous sulfate. Specific objectives were: (1) to use the INT assay to predict the dosage

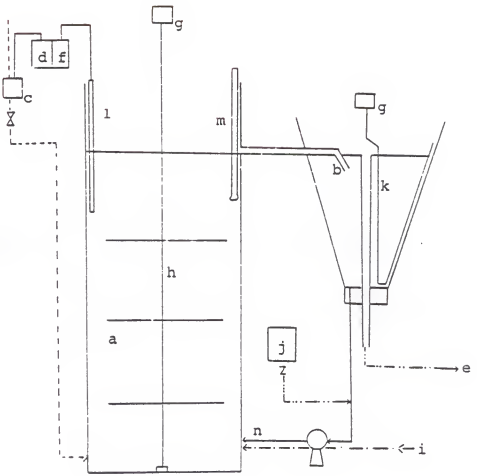
of ferrous sulfate which would be effective in controlling bulking, (2) to compare the inhibition of filaments to inhibition of overall sludge biomass caused by the toxicant, and (3) relate trends in specific activity of filaments to trends in SVI.

6.2. Materials and Methods

6.2.1. Activated Sludge System

The activated sludge system consisted of a cylindrical aeration basin and conical secondary clarifier having liquid volumes of 10.0 L and 3.0 L, respectively (Fig. 6-1). The aeration basin was stirred at 180 rev/min by 3 sets of paddles affixed to a motor-driven shaft. The dimensions of the paddles are shown in Figure 6-2. Pure oxygen was supplied to the aeration basin through a cotton filter and was sparged via porous stone diffusers. The oxygen flow was controlled by a solenoid valve operated by a DO recorder/controller (Rustrac 288/F205, Gulton) and analyzer (800/DO-40, New Brunwick Scientific) to achieve a preset DO concentration. The range of DO was normally $\pm$ 2% of the setpoint. The mean velocity gradient achieved by stirring was 85 s^{-1}. A neglible amount of turbulence was introduced via the oxygen sparging.

The secondary clarifier was fitted with a wire wall-scraper rotated at 1 rev/min to aid the movement of consolidating sludge to the recycle line. The rate of recycle was 100% of the influent flow. Sludge was wasted directly from the aeration basin on a once daily basis. Feed was domestic wastewater collected from the



a : aeration basin
b : secondary clarifier
c : air pump or solenoid valve (when pure oxygen used)
d : DO recorder-controller
e : effluent
f : DO analyzer
g : stirrer motor
h : stirrer
i : influent
j : inhibitor feed tank
k : scraper
l : DO electrode
m : thermometer
n : return sludge line

Figure 6-1. A schematic diagrm of bench-scale activated sludge system

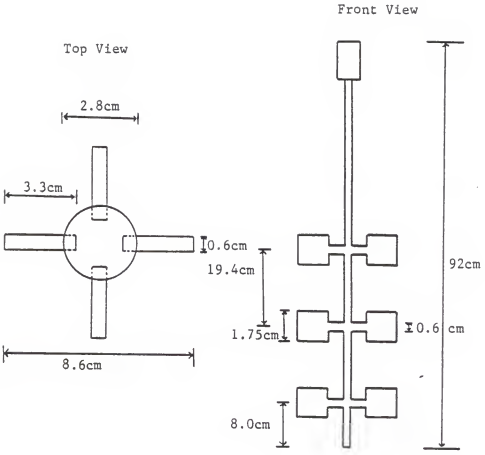


Figure 6-2. Paddles diagram

headworks of the University of Florida's wastewater treatment plant. The sewage was settled overnight before use. Influent wastewater and return sludge were dosed continuously via a peristaltic pump. Concentrations of COD in the sewage feed were increased by adding sodium acetate (0.2-0.4 kg/m^3). The system was operated at ambient (outdoor) temperature, which varied from 19°C to 29°C.

6.2.2. Toxicity Bioassay

One litre of activated sludge was withdrawn from the aeration basin and brought to the laboratory. The sample was aerated for 30 min before the toxicity bioassay in order to strip out volatiles (e.g., H_2S) and maintain aerobic conditions. Known amounts of ferrous sulfate in the range of 0-40 g/m^3 as Fe were added to 100 mL aliquots in 250 mL Erlenmeyer flasks. Treated aliquots were agitated for 2 hours at 100 oscillatios/min on a shaker table (model 3590, Lab Line). pH was adjusted to 7.6 before transfering duplicate 5 mL aliquots from each flask into vials for INT-dehydrogenase assay. The ferrous sulfate concentration to inhibit 50% of filament dehydrogenase activity (DHA_c) of samples (IC_{50}) was determined as follows. The degree of inhibition at each Fe^{2+} dose was determined by comparing the DHA_c of the experimental sample to the DHA_c of the control. The degree of inhibition was transformed according to the following equation (Johnson et al., 1974):

$$\Gamma = \frac{\text{\% inhibition}}{100 - \text{\% inhibition}} \qquad (6\text{-}1)$$

A plot of Γ versus dosage approximated a linear relationship. Lines of best fit to the transformed data were determined by least squares linear regression. The IC_{50} was taken as the dosage at which the best fit relationship gave a value of Γ equal to 1.0. The dosage applied to the pilot plant was one-fourth to one-half of the IC_{50}.

6.2.3. Dehydrogenase Activity

Fresh sludge samples were aerated for 30 min and pH was adjusted to 7.6 prior to the dehydrogenase assay. Duplicate 5 mL aliquots of the activated sludge sample were amended with 0.5 mL of 0.2% INT (Eastman Kodak). The mixtures were incubated in the dark at room temperature (22 $\pm$ 2°C). After 20 min, the reaction was stopped by adding 0.5 mL of 2.4N HCl. INT-dehydrogenase activity of overall sludge biomass (DHA_e) was determined as follows. 1-6 mL of the INT-treated mixture (depending on color intensity) was pipetted into a centrifuge tube and centrifuged at 1500 x g for 10 min. After carefully decanting the supernatant, 5 mL of dimethylsulfoxide (DMSO) was added to the centrifuge tube and the tube was capped. The pellet was resuspended by vortexing for 15 s and extraction of INTF allowed to continue in darkness for 15 min. Optical density of the supernatant was determined by absorption spectrophotometery (Bauch and Lomb Spectronic 70, 1 cm

path length) at 465 nm. The following equation (see Chapter 4 for definitions of terms) was used for calculating INT-dehydrogenase activity.

$$DHA = \frac{1002\ D_{465}\ v}{VCtF} \qquad (6\text{-}2)$$

The relative INT-dehydrogenase activity of filamentous microoganisms (DHA_c) was determined by microscopic observation of prepared specimens (Bitton and Koopman, 1982). Smears were made of the INT-treated mixture and allowed to air dry. The dry smears were covered with 0.05% (w/v) aquous solution of malachite green (Allied Chemical) for 1 min. Malachite green was drained completely off the slides and the smears were air dried prior to examination under oil emulsion at 1000x using bright field microscopy. Counts were made of active (filaments with red INTF crystals present) and non-active (INTF crystals absent) lengths of filaments. The length of all filaments associated with three flocs chosen at random on each of 3 slides were counted in terms of the number of 0.7 μm long segments of filament which were considered either active or inactive. DHA_c was calculated according to:

$$DHA_c,\ \% = \frac{\text{Length of active filaments}}{\text{Total filament length}} \times 100 \qquad (6\text{-}3)$$

6.2.4. Total and Soluble Iron

Soluble iron was measured daily during the course of the pilot plant operation by filtering 20 mL of effluent through a

0.45 μm membrane filter (Gelman GN-6). Iron was maintained in solution by adding 0.5 mL of concentrated HCl to the filtrate. Total soluble iron was measured by adding 2 mL of concentrated HCl to 10 mL of mixed liquor and allowing the sample to stand at room temperature for one week. This method was modified and confirmed (deviation of results was within ± 2%) according to the method described by Chang (1979). The sample was then filtered through 0.45 μm membrane filter prior to analysis. The analyses were made with a Perkin-Elmer Model 5000 atomic absorption spectrometer using an air-acetylene flame. A certified atomic absorption iron solution (Fisher, A.C.S) was used to prepare standards.

6.2.5. Identification of Filamentous Microorganisms

Eikelboom's key (1975, 1977) as modified by Strom and Jenkins (1984) was used to identify filamentous microorganisms to type. The microscope was a Nikon Optiphot with a 35 mm camera attachment and autoexposure accessory. Gram stain, modified Neisser's stain, intracellular sulfur granules test, and sheath stain (Richard et al., 1981) were conducted as part of the identification procedure. The procedures for these stains are given in Appendix A.

6.2.6 Other Analytical Methods

F/M was calculated according to the following equation (Metcalf and Eddy, 1979):

$$F/M = \frac{Q\ \Delta COD}{V\ X_1} \qquad (6\text{-}4)$$

where F/M is expressed as g COD removed/g VSS d, Q = influent wastewater flowrate (m^3/d), ΔCOD = (influent total COD - effluent filterable COD) (g/m^3), V = volume of aeration basin (m^3) and X_1 = VSS concentration of activated sludge in aeration basin (g/m^3). F/M was controlled by the amount of sludge wasted, the addition of sodium acetate and wastewater flowrate. COD, TSS, and VSS were measured according to Standard Methods (APHA, 1980), methods 508A, 209D and 209E, respectively. Settling characteristics of activated sludge were assessed using the diluted sludge volume index (Lee et al., 1983). Two or three 1-L graduate cylinders were set up for a series of two-fold dilutions of the activated sludge based on the prior knowledge of the settleability of the sludge. The graduate cylinders were stirred individually for 30-60 seconds using a plunger to resuspend the solids. The settled volume after 30 min (SV_{30}) was observed where the settled volume was less than and closest to 200 mL. The diluted SVI was calculated using:

$$\text{DSVI (mL/g)} = \frac{SV_{30}\ \text{(mL/L)} \times 2^n}{SS\text{(g/L)}} \qquad (6\text{-}5)$$

where n is the number of two-fold dilutions required to obtain a settled volume (SV_{30}) less than 200 mL and SS is the suspended solids concentration of the activated sludge before dilution.

6.3. Results and Discussion

6.3.1. Overview of Experiments

Two experiments were carried out. The first was performed with severely bulking sludge (SVI $\geq$ 400 mL/g). Toxicant was added at first to the aeration basin and later to the return activated sludge line. The second experiment was conducted with a low target value of SVI based on the experience from experiment 1. Toxicant was added to the aeration basin. F/M in experiment 2 was more carefully controlled than in experiment 1. The content of iron in effluent and mixed liquor was measured during experiment 1 in order to determine the fate of iron added. Filament types present in activated sludge were identified during both experiments.

6.3.2 Experiment 1

6.3.2.1. Startup and initial development of bulking sludge

The experiment was begun with seed activated sludge from the campus treatment works at the University of Florida (UF). Target values for dissolved oxygen and F/M were 0.5 g/m^3 and 0.6 g COD/g VSS-d, respectively. The DSVI at days 13 and 14 was 400 and 320 mL/g, respectively (Fig. 6-3). The DHA_c on days 13, 14 and day 15 was high (81-86%). The DSVI reached 1320 mL/g TSS on day 15.

6.3.2.2. First application of ferrous sulfate

The IC_{50} of bulking sludge, based on DHA_c, at this time (day 14) was 16 g/m^3 Fe^{2+} (16.7 mg Fe^{2+}/g TSS at a sludge TSS of 960

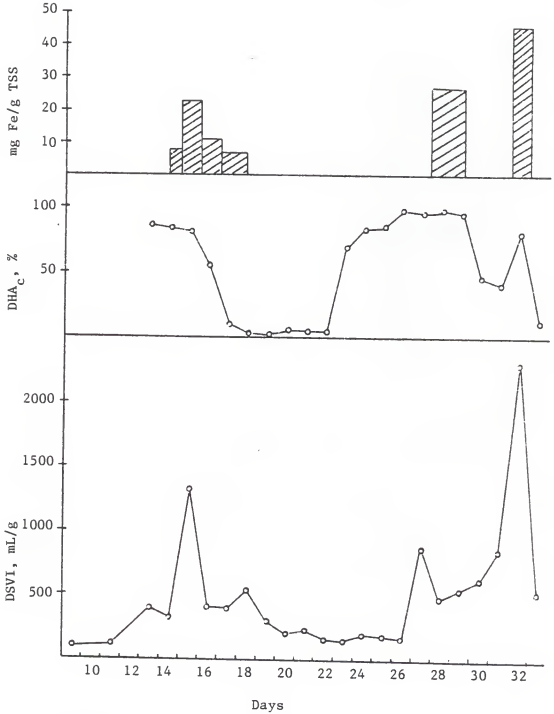


Figure 6-3. Temporal variation of DHA_c and SVI in relation to Fe^{2+} dosage, Experiment 1

g/m^3) as shown in Figure 6-5. On day 14, a dose equal to 8 g of ferrous sulfate as Fe per cubic metre of aeration basin volume (one-half the IC_{50}) was added to the aeration basin three times at equal intervals over the course of the day. The dosage on day 15 was increased to 16 g/m^3. Exposure frequency was 3.0 day^{-1}. The dosage was reduced to 8 g/m^3 on day 16 because the DHA_c had dropped to 54%. Toxicant additions were continued for two more consecutive days (during day 16, 17, and 18) in order to further decrease DSVI. The frequency of addition on days 16, 17 and 18 was 3.0 day^{-1}. When the DHA_c was less than 10% (after day 19), the DSVI dropped below 200 mL/g. The DHA_e was also decreased by toxicant additions, but it was not nearly as sensitive to iron additions as DHA_c (Fig. 6-4). DHA_e was correlated with F/M; for example, both parameters reached peak values on day 16.

Filamentous organisms were present in abundant amounts on day 13 (Table 6-1). The DSVI, F/M and effluent FCOD on this day were 400 mL/g TSS, 0.44 g COD/g VSS d and 33 g/m^3, respectively. The DO was 0.63-0.78 g/m^3. Thiothrix II was the No. 1 ranked filament, present at abundant to excessive amounts. Sphaerotilus natans was ranked No. 2, at abundant concentration. Type 0041 was present in minor amounts. Micrographs of the activated sludge are shown in Fig. 6-6. Filament communities observed in the activated sludge during days 8 through 13 were relatively stable in terms of species composition. Types 0041, 021N and 1701 appeared as minor components. The effect of ferrous sulfate on bulking sludge is

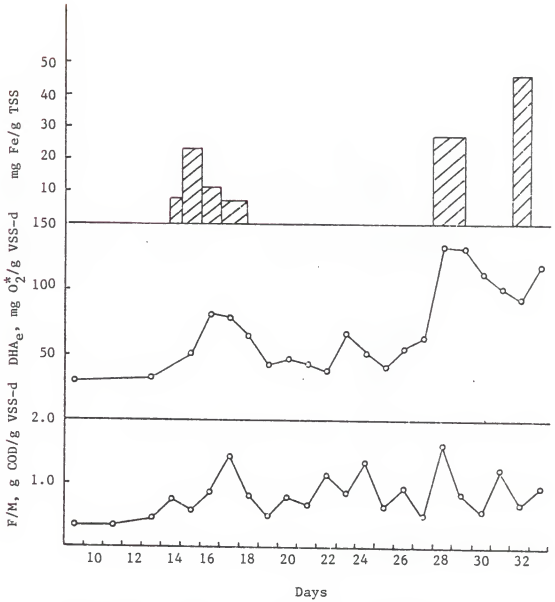


Figure 6-4. Temporal variation of DHA_e and F/M in relation to Fe^{2+} dosage, Experiment 1

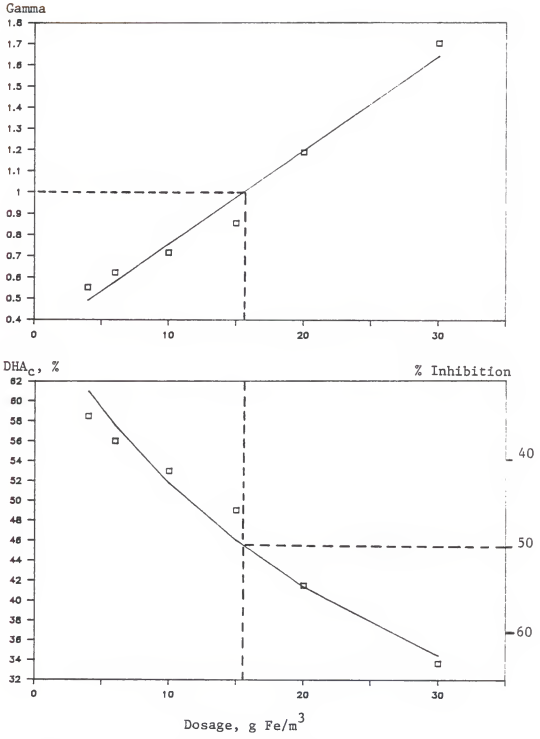


Figure 6-5. Estimation of IC_{50} of ferrous sulfate for the first-bulking ocurrence based on DHA_c, Experiment 1. (TSS of sludge = 0.96 g/L)

Table 6-1. Identification of filamentous microorganisms present in activated sludge on day 13, experiment 1.*

	Filament # A	B	C
Branching	+,False	-	-
Motility	-	-	-
Filament Shape	straight	smoothly-curved	straight
location	ext. from floc	ext. from floc	in floc
Crosswalls	+	+	+
Sheeth	+	+,thin	+
Attached unicells	-	-	+/-
Filament length (μm)	50-200	20-200	50-100
diameter	1.2-1.4	0.8-1.2	1.6-1.8
Cell shape	round-end rods	rectangles	squares
size (μm)	1.2-1.4x2	1.0x1.5	1.6x2.0
Sulfur Deposits	-	-	-
Other Granules	+/-	+/-	-
Gram Stain	-	-	+
Neisser Stain	-	-	-
Commonness	Abundant	Ab. to Excessive	Some
Rank	2	1	3
Identification	Sphaerotilus natans	Thiothrix II	Type 0041

*This table was provided by Dr. Michael Richard, Colorado State University.

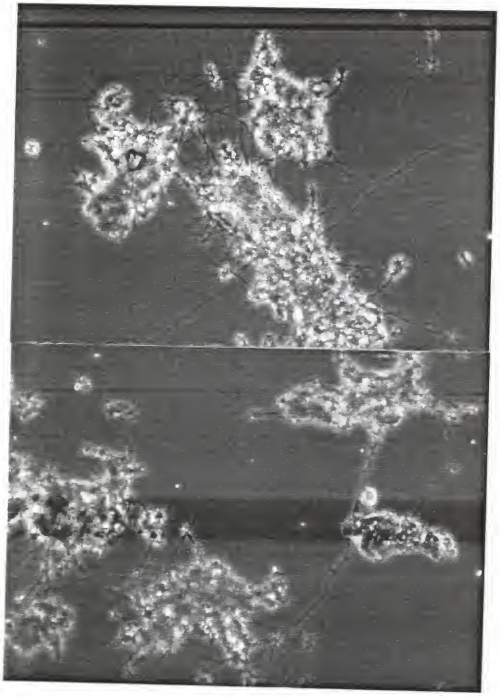


Figure 6-6. Micrographs of activated sludge, day 13
100x, phase contrast.
bright spots: sulfur granules
bar = 100 m

shown in Fig. 6-7, which consists of a series of micrographs taken on the first, second and third days following the initiation of iron dosage.

The content of iron in effluent was measured in terms of iron distribution by the addition of ferrous sulfate. The average value of filterable-iron content before toxicant addition was 0.3 g/m^3 (Fig. 6-8). During or after dosing toxicant, the average concentration of iron in the effluent was 0.74 g/m^3, except on day 15 (Fe = 4.3 g/m^3), which was the first day after addition of ferrous sulfate began. The effluent iron concentration fell back to 0.3 g/m^3 three days after toxicant addition was terminated. The total concentration of iron added over the 4 day period was 40 g/m^3 based on the volume of the aeration basin, which averages 10 g/m^3 per day. The range of iron content in mixed liquor was 15.9 - 53.7 mg Fe/ g TSS on a dry weight basis. The iron concentration in mixed liquor went up to 36.5 g/m^3 on the day after initial dosing of ferrous sulfate and averaged 22 g/m^3, with a range of 13 g/m^3 - 36 g/m^3.

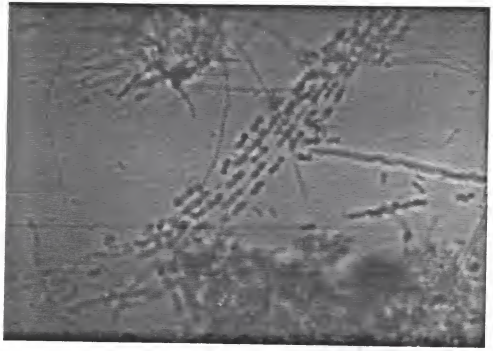
6.3.2.3. Recurrence of bulking and second application of ferrous sulfate

On day 24, filamentous microorganisms were very active (DHA_c = 85%), the number of filaments was small, and the DSVI was 199 mL/g (Fig. 6-3). The DHA_c of filamentous organaisms reached almost 100% on day 27 corresponding to a sharp rise in DSVI. Sphaerotilus natans was identified as the predominant organism whereas Thiothrix II was ranked second with a "very common"

A

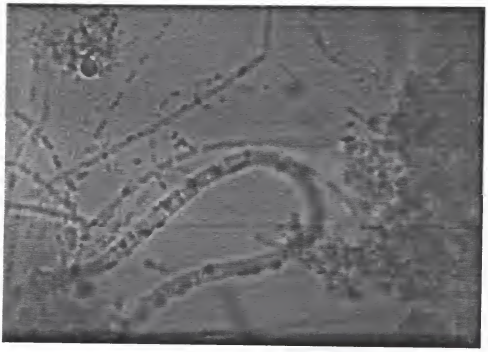
Figure 6-7. Effect of ferrous sulfate on INTF content of filamentous microorganisms in bulking sludge. (All micrographs taken at 1000x using bright-field illumination)
A - after 1 day of Fe^{2+} addition (day 15)

B



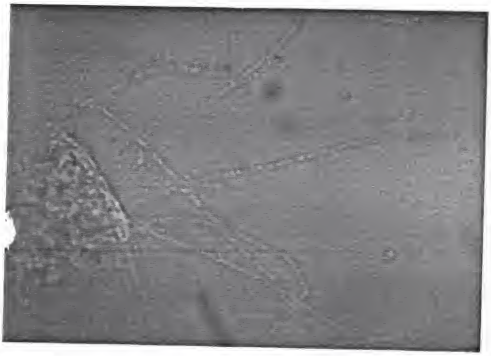
B - After 2 days of Fe^{2+} addition (day 16)
Figure 6-7 (continued)

C



C - After 3 days of Fe^{2+} addition (day 17)
Figure 6-7 (continued)

D



D - After 5 days of Fe^{2+} addition (day 19)
Figure 6-7 (continued)

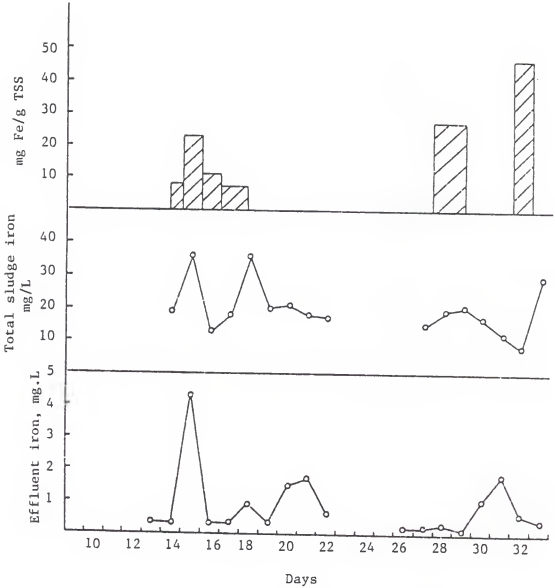


Figure 6-8. Temporal variation of total and filterable effluent iron concentration in relation to Fe^{2+} dosage

abundance (Table 6-2). Type 0041 was common and some Nocardia sp. were observed. The size of flocs ranged between 50 and 300 μm. A toxicity bioassay on activated sludge taken from the aeration basin on day 27 was carried out to determine the IC_{50}. The IC_{50} was estimated to be 23 g/m^3 Fe^{2+} (23 mg Fe/g TSS based on a sludge TSS of 1.0 g/m^3) as shown in Figure 6-9. The iron dosing parameters were calculated as follows:

a. Dosed iron concentration at the iron dose point (C)

$C = M/R = 9.4$ mg Fe/L

where M = iron application rate, 0.3 g Fe/d

R = flow of stream (RAS) into which iron is dosed, 32 L/d

b. Exposure frequency of activated sludge to iron dosing (f)

$$f = \frac{Q + R}{V} = 6.4\ d^{-1}$$

where Q = flow of influent, 32 L/d

V = volume. 10 L

Ferrous sulfate was dosed at 9.4 g Fe/m^3 (40% of the IC_{50}) into the return activated sludge (RAS) line by pumping for 1.7 days continuously. Exposure frequency of activated sludge to Fe dosing was 6.4 day^{-1}. The DHA_c of the sludge fell to 46% one day after toxicant addition was stopped, although the SVI remained high (500 mL/g). The change of SVI and DHA_c was observed during the next two days. More severe bulking occurred as the DHA_c of filamentous organisms rose to 81% on day 32. Addition of ferrous sulfate was

Table 6-2. Identification of filamentous microorganisms present in activated sludge on day 27, experiment 1.*

	Filament # A	B	C	D
Branching	+,False	-	-	+
Motility	-	-	-	-
Filament Shape	straight	smoothly-curved	straight	mycelial
location	ext. from floc	ext. from floc	in floc	free
Crosswalls	+	+	+	+
Sheeth	+	+/-	+	-
Attached unicells	-/+	-	+	-
Filament length (μm)	200->500	100-200	50-300	10-20
diameter	1.4-1.8	1.0-1.2	1.6-1.8	1.0
Cell shape	round-end rods	rectangles	squares	irregular
size (μm)	1.4-1.8x2.5	1.0x2.0	1.8x2.0	1.0x2.0
Sulfur Deposits	-	+	-	-
Other Granules	+/-	-	-	+
Gram Stain	-	-	+	+
Neisser Stain	-	-	-	-,+granules
Commonness	Abundant	very common	common	few
Rank	1	2	3	4
Identification	Sphaerotilus natans	Thiothrix II	Type 0041	Nocardia

*This table was provided by Dr. Michael Richard, Colorado State University.

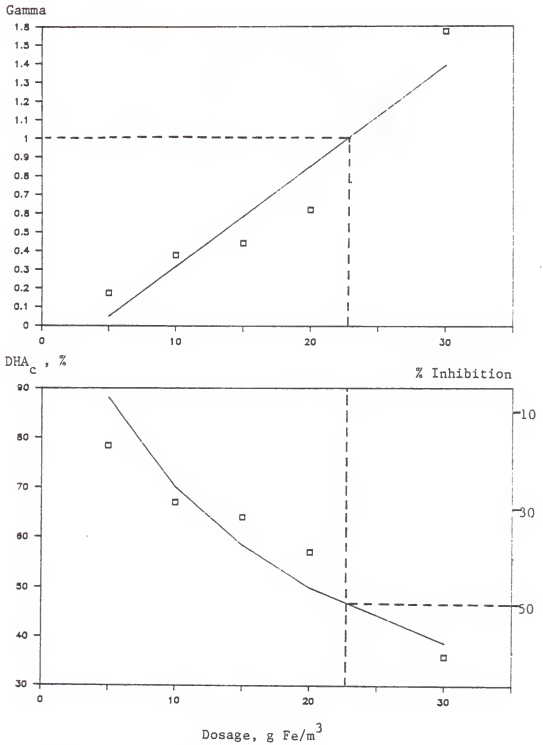


Figure 6-9. Estimation of IC_{50} of ferrous sulfate for recurrence of bulking based on DHA_c, Experiment 1. (TSS of sludge = 1.00 g/L)

started again the next day. 30 g Fe/m^3 (46.1 mg Fe/g TSS) was added into the aeration basin at 3.0 day^{-1} frequency. The SVI and DHA_c fell in tandem in response. This result indicates that toxicant addition must continue beyond the time that DHA_c falls to 40%. During the recurrence of bulking, DHA_c showed a earlier trend of increase than DSVI and DHA_e was not affected as much as DHA_c by the addition of iron.

6.3.2.4. General discussion

It is apparent from Fig. 6-3 that ferrous sulfate had a dramatic effect on DHA_c. In comparison, it can be seen from Fig. 6-4 that DHA_e was affected to a much lesser extent. At day 19, the DHA_c was near zero, after which SVI fell to satisfactory levels. Based on the observed results it would appear that the dosing of toxicant can be stopped after DHA_c falls to 10% or below, even if the SVI has not yet fallen below the target value. In this case, the SVI was 292 mL/g at the time iron dosages were terminated, yet the SVI continued dropping to below 200 mL/g.

The bulking problem caused by Thiothrix and S. natans in this experiment was probably due to relatively low DO (0.6-0.7 g/m^3) and septic wastewater. The septic wastewater possibly resulted from washing sewer pipes and was recognized by the black color of the raw sewage on days 15 and 28. Less iron was required to control bulking in the first instance (cumulative 40 g/m^3 in 4 days) than in the second instance (52 g/m^3 during 1.7 days). This result is consistent with the results of the two toxicity

bioassays which were conducted. The IC_{50} (23 g/m^3) for the second case was higher than that (16 g/m^3) in the first case. This can perhaps be related to the types of filamentous microorganisms present. Previously, Kim et al. (1987) found Thiothrix (the predominant filament in the first instance) to be more sensitive to oxidants than S. natans (the predominant filament in the second case). It was shown in chapt. 5 of this dissertation that pure cultures of Thiothrix were more sensitive to ferrous sulfate than S. natans.

6.3.3. Experiment 2

6.3.3.1. Startup and development of bulking sludge

Experiment 2 was started in the same way as experiment 1. F/M and dissolved oxygen concentration were controlled in the range of 0.53-0.72 g COD/g VSS-d and 0.62-0.71 g/m^3 during the one month period of experiment 2. Bulking sludge was developed a total of three times.

6.3.3.2. First addition of ferrous sulafte

The filament types present during the first bulking episode, as identified on days 7 and 16, were Sphaerotilus natans, type 1701, Thiothrix II and type 0041 (Tables 6-3 and 6-4). Sphaerotilus natans and Thiothrix II were predominant. A toxicity bioassay of the sludge, conducted on day 7, gave an IC_{50} of 12.0 g Fe^{2+}/m^3 (8.3 mg Fe^{2+}/g TSS based on a sludge TSS of 1450 g/m^3) as shown in Fig. 6-12. One third of IC_{50} (4 g/m^3) was dosed to the aeration basin manually three times a day beginning on day 7, when

Table 6-3. Identification of filamentous microorganisms present in activated sludge on day 7, experiment 2.*

	Filament # A	B	C	D
Branching	+, False	-	-	+
Motility	-	-	-	-
Filament Shape	straight	smoothly-curved	straight, bent	irregular
location	ext. from floc	ext. from floc	in floc	in floc
Crosswalls	+	+	+	+
Sheeth	+	+/-	+	-
Attached unicells	-	-	+/-	-
Filament length (μm)	50-300	30-120	20-60	10-20
diameter	1.2-1.4	0.7-1.0	0.8	1.0
Cell shape	oval-end rods	rectangles	oval-end rods	irregular
size (μm)	1.2-1.4x2.3	0.7-1.0x1-2	0.8x1.5	1.0x2-4
Sulfur Deposits	-	+	-	-
Other Granules	-	+	++	+
Gram Stain	-	-	-	+
Neisser Stain	-	-	-	-,+granules
Commonness	common	very common	some	common
Rank	2	1	4	3
Identification	Sphaerotilus natans	Thiothrix II	Type 1701	Nocardia sp.

*This table was provided by Dr. Michael Richard, Colorado State University.

Table 6-4. Identification of filamentous microorganisms present in activated sludge on day 12, experiment 2.*

	Filament # A	B	C	D
Branching	+, False	-	-	-
Motility	-	-	-	-
Filament Shape	straight	smoothly-curved	straight	straight, bent
location	ext. from floc	ext. from floc	in floc	in floc
Crosswalls	+	+	+	+
Sheeth	+	+	+	+
Attached unicells	-	-	+/-	+/-
Filament length (μm)	50-300	40-100	50-150	20-40
diameter	1.2-1.4	0.7-1.0	1.6	0.8
Cell shape	oval-end rods	rectangles	squares	round-end rods
size (μm)	1.2-1.4x2.3	0.7-1.0x1.2	1.6x1.2	0.8x1.5
Sulfur Deposits	-	+, few	-	-
Other Granules	-	+	-	++
Gram Stain	-	-	+	-
Neisser Stain	-	-	-	-
Commonness	some	some	few	some
Rank	1	2	4	3
Identification	Sphaerotilus natans	Thiothrix II	Type 0041	Type 1701

*This table was provided by Dr. Michael Richard, Colorado State University.

the DSVI was 191 mL/g (Fig. 6-10). 2.75 - 3.31 Fe mg/g TSS of dose were continued between day 7 and day 11. Exposure frequency was 3.0 d^{-1}. Previously, in experiment 1, poor results such as losses of solids occurred when the DSVI was allowed to exceed approximately 300 mL/g before iron dosing was initiated, therefore a lower target value (200 mL/g) was established in this experiment. During the first day of iron dosing, DHA_c dropped from 85% to 24% and DSVI began to decline. Iron application was continued until day 11, at which DHA_c fell to 16%. DSVI continued to decline until day 14, when a minimum value of 138 mL/g was reached. DHA_e was increased as DSVI showed a increasing trend and decreased down to 67 mg O_2*/g VSS-d on day 11 following iron addition. However, DHA_e was less sensitive to iron addition than DHA_c during the first bulking episode.

6.3.3.3 Recurrence of bulking and second addition of ferrous sulfate

As DHA_c increased from 9% on day 14 to 42% on day 15, DSVI began rising again, reaching 233 mL/g on day 16. Application of iron was resumed at this time. A bioassay conducted on day 16 (Fig. 6-13) gave a DHA_c-based IC_{50} of 24.0 g/m^3 (17.9 mg Fe^{2+}/g TSS based on a sludge TSS of 1340 g/m^3). The iron dose added to the aeration basin was one-third of the IC_{50} (8 g/m^3). The frequency of exposure on succesive days (from 10:00 AM day 16 to 10:00 AM day 20) was 3 day^{-1}. This dosage was successful in arresting and subsequently reversing the upward trend in DSVI (Fig. 6-10). Iron application was discontinued on day 20, when

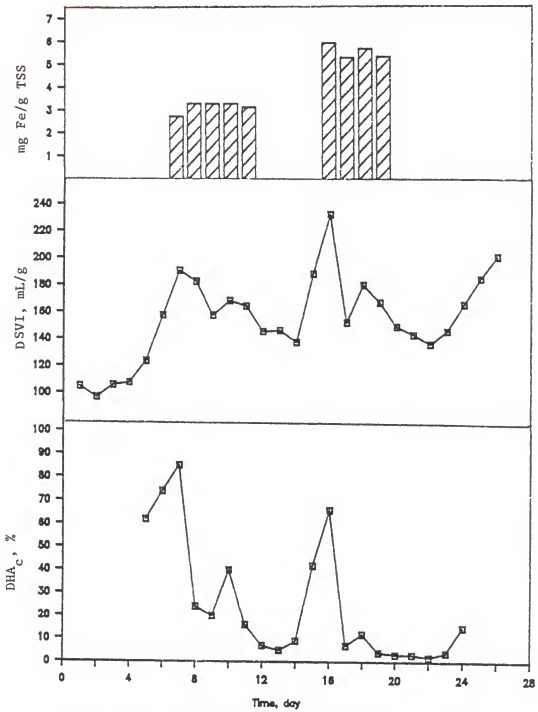


Figure 6-10. Temporal variation of DHA_c and SVI in relation to Fe^{2+} dosage, Experiment 2

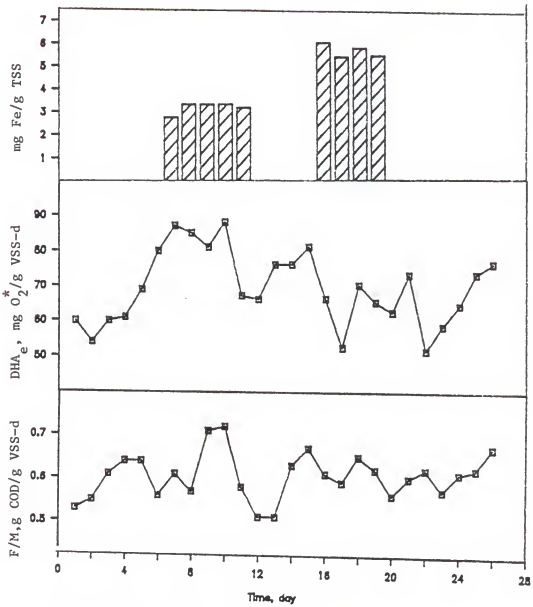


Figure 6-11. Temporal variation of DHA_e and F/M in relation to Fe^{2+} dosage, Experiment 2

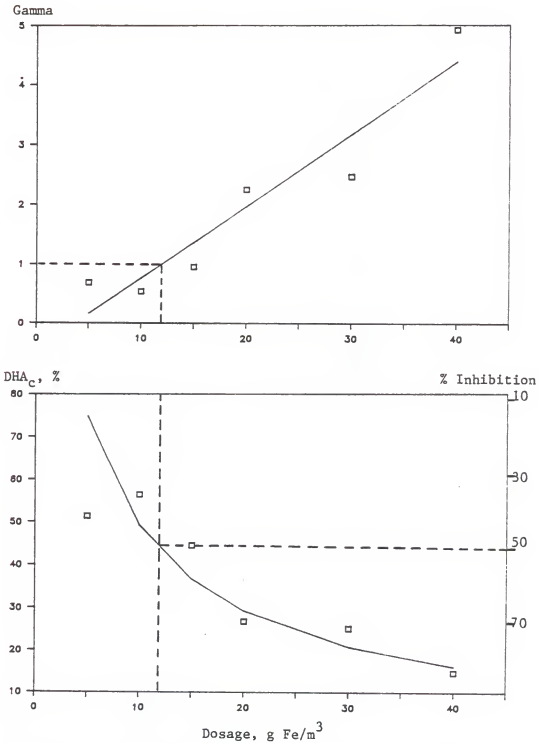


Figure 6-12. Estimation of IC_{50} of ferrous sulfate for the first instance based on DHA_C, Experiment 2. (TSS of sludge = 1.45 g/L)

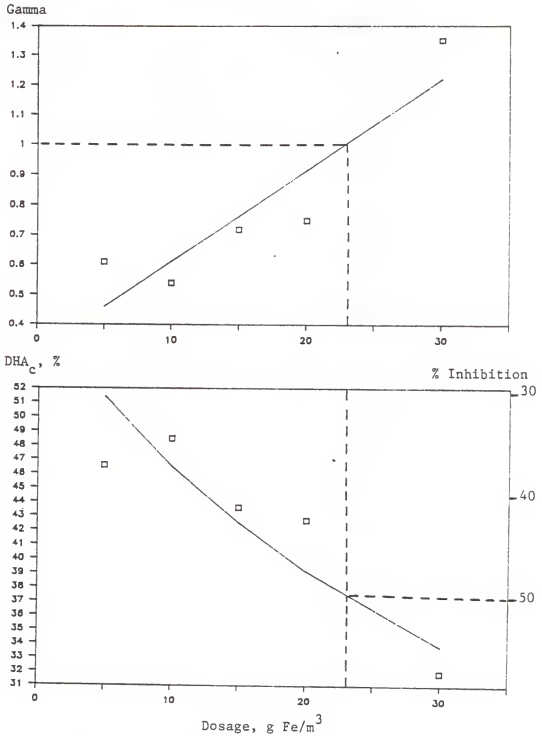


Figure 6-13. Estimation of IC_{50} of ferrous sulfate for recurrence of bulking baced on DHA_c, Experiment 2 (TSS of sludge = 1.34 g/L)

DSVI was 150 mL/g and DHA_c was 3 %. After that, DSVI remained below 150 mL/g for 4 days. During the second episode of bulking, the predominant filament types were Sphaerotilus natans, Thiothrix II, and Type 0041, as observed on days 16 and 20 (Tables 6-5 and 6-6). Type 1701 was absent. This assemblage suggests that low DO and septic wastewater were the primary causes of bulking. The sample observed on day 20 contained fewer filamentous organisms (abundance = "some") than the sample observed on day 16 (abundance = "very common"), which is consistent with the trends in DSVI. The increasing trend of DHA_e was controlled by toxicant additions as shown in Figure 6-11.

6.3.3.4 Final recurrence of bulking

Final bulking sludge was obtained after several days of contining operation under the same conditions, which was also expected by the increase of DHA_c on the day 24 and the increasing trend of DHA_e after day 23.

6.3.3.5. General discussion

Filament types observed in mixed liquors of the samples (samples from day 7 and day 16 are of bulking activated sludge; samples from day 12 and day 20 are of non-bulking activated sludge, as shown in Tables 6-3 to 6-6, were stable in terms of species composition. F/M was controlled in the range of 0.53-0.72 g COD/g VSS-d (Figure 6-11) and dissolved oxygen maintained under the concentration of 0.62-0.71 g/m^3. This condition caused a development of low DO bulking. Strom and

Table 6-5. Identification of filamentous microorganisms present in activated sludge on day 16, experiment 2.*

	Filament # A	B	C	D
Branching	+,False	-	-	+
Motility	-	-	-	-
Filament Shape	straight	smoothly-curved	straight	irregular
location	ext. from floc	ext. from floc	in floc	in floc
Crosswalls	+	+	+	+
Sheeth	+	+/-	+	-
Attached unicells	-	-	+	-
Filament length (μm)	100-300	50-150	50-150	10-20
diameter	1.2	0.6-1.0	1.8	1.0
Cell shape	oval-end rods	rectangles	squares	irregular
size (μm)	1.2x2.0	0.6-1.0x1.2	1.8x1.5-2	1.0x2-4
Sulfur Deposits	-	-	-	-
Other Granules	-	-	-	+
Gram Stain	-	-	+/-	+
Neisser Stain	-	-	-	-
Commonness	common	common	some	some
Rank	1	2	3	4
Identification	Sphaerotilus natans	Thiothrix II	Type 0041	Nocardia sp.

*This table was provided by Dr. Michael Richard, Colorado State University.

Table 6-6. Identification of filamentous microorganisms present in activated sludge on day 20, experiment 2.*

	Filament # A	B	C	D
Branching	+,False	-	-	
Motility	-	-	-	
Filament Shape	straight	smoothly-curved	straight,	
location	ext. from floc	ext. from floc	in floc	
Crosswalls	+	+	+	
Sheeth	+	+/-	+	
Attached unicells	-	-	+	
Filament length (μm)	100-300	30-150	50-100	
diameter	1.2	0.6-1.0	1.6	
Cell shape	round-end rods	rectangles	squares	
size (μm)	1.2-2-2.5	0.6-1.0x1-2	1.6x1.5	
Sulfur Deposits	-	-	-	
Other Granules	-	-	-	
Gram Stain	-	-	+(weak)	
Neisser Stain	-	-	-	
Commonness	some	some	few	
Rank	1	2	3	
Identification	Sphaerotilus natans	Thiothrix II	Type 0041	

*This table was provided by Dr. Michael Richard, Colorado State University.

Jenkins (1984) associated the occurrence of S. natans or type 1701 in activated sludge with a DO concentration in the mixed liquor that was too low for the applied organic load. Thiothrix spp. and type 021N were considered as tentative indicators of low DO conditions. Because all four of these filament types were present at one time or another, it is probable that DO limitation did play a contributing role in bulking episodes. Filaments observed in the non-bulking activated sludge, after toxicant addition contained more empty sheaths. DHA_e averaged 61 mg O_2*/g VSS-d before bulking problem occurred and increased up to 87 % as filamentous microorganisms were growing abundantly. While iron was being applied DHA_e was reduced to 67 % at day 11 and incresed as DSVI began to rise again (Fig. 6-11). However DHA_e was affected by iron to a much lesser extent than was DHA_c. The specific activity (DHA_c) parameter consistently anticipated trends in DSVI once iron application was begun. Successful control of bulking was achieved by adding ferrous sulfate (one third of IC_{50}, and exposure frequency = 3.0 d^{-1}) into the aeration basin for 4-5 consecutive days until DHA_c fell below 10 %.

CHAPTER 7
EFFECT OF SUSPENDED SOLIDS CONCENTRATION ON TOXICITY
OF FERROUS SULFATE TO BULKING ACTIVATED SLUDGE

7.1 Introduction

Chlorination has been used occasionally for bulking control in the USA for many years (Smith and Purdy 1936, Tapleshay 1945). Recently its use has become widespread in the USA (Jenkins et al. 1982, Neethling et al. 1985). However, the effectiveness of this method varies with different filamentous organisms and with some unknown factors. During the period of chlorination the effluent is sometimes turbid (Smith and Purdy, 1936; Tapleshay, 1945).

Waitz and Lackey (1959) observed that growth of Sphaerotilus was inhibited by a concentration of 25 g/m^3 (as Fe) of ferric chloride. In a laboratory study, the addition of ferric chloride to the aeration tank has been reported not only to reduce sludge bulking, but also to increase the organic removal efficiency (Pfeffer 1967, Carter and McKinney 1973). Rensink (1979) achieved good results by continuous dosing of ferrous sulfate to the aeration tank in a completely mixed plant with bulking sludge. Chang et al. (1979) surveyed the inhibitory effect of selected iron compounds such as Fe-cystine and ferrous sulfate. They found these compounds to be highly effective in inhibiting the growth of Sphaerotilus and hence considered them to

be candidate compounds for controlling Sphaerotilus bulking. Although Fe-cystine had the higher inhibitory value, ferrous sulfate has greater practical potential from an economic point of view. One of the least costly sources of ferrous sulfate is the waste pickle liquor from steel plants. In the present research (see chapt. 5), it was found that the order of sensitivity of pure cultures to ferrous sulfate was Thiothrix, type 021N and Sphaerotilus natans (from most to least sensitive).

Though the use of ferrous sulfate to control bulking has a potential advantage over other methods, because as stated above, it can improve organic removal efficiency while reducing sludge bulking. A full scale plant study to evaluate ferrous sulfate for phosphorous removal was performed by Hogge et al.(1985). Suspended solids removal in secondary clarification process was enhanced by ferrous sulfate addition and significant improvements in sludge thickening and dewatering processes resulted from ferrous sulfate addition. However, this method is not yet completely understood with respect to several factors influencing the inhibition of filamentous microorganisms and its mechanism of iron inhibition. Two important factors which must be considered when applying ferrous sulfate for bulking control are the suspended solids concentration and pH of activated sludge at the point of contact. The effect of pH on toxicity of ferrous sulfate to Sphaerotilis natans was examined previously in this research (see chapt. 5). Suspended solids varies by a factor of two or

more between the mixed liquor and return sludge, which are the most common points of toxicant addition. In certain cases, application of chemicals to the return sludge may be ineffective because of insufficient exposure frequency (Jenkins et al. 1982, Neethling et al. 1985). The objective of the research presented in this chapter was to determine the effect of total suspended solids concentration on the toxicity of ferrous sulfate to bulking activated sludge.

7.2. Materials and Methods

7.2.1 Activated Sludge Pilot Plant

A bench scale pilot plant was operated to provide bulking sludge for use in toxicity tests. The pilot plant was seeded with activated sludge from the campus treatment works at the University of Florida (UF). Diluted sludge volume index (DSVI) (Lee et al., 1983) and filament community composition were monitored daily. Operation was continued until an SVI of 205 ml/g was reached. Sludge was then sacrificed for use in toxicity tests.

The pilot plant consisted of a cylindrical, 10 L aeration basin and conical, 3.0 L secondary clarifier. Mixing in the aeration basin was by paddles. Oxygen was supplied through porous stone diffusers. Dissolved oxygen was controlled by a feedback system consisting of an oxygen electrode and DO analyzer (800/DO 40, New Brunswick Scientific) and recorder/controller (Rustrak 288/F205, Gulton).

Feed for the pilot plant was domestic sewage obtained from the headworks of the UF treatment facility. The sewage was settled overnight before use. Influent sewage and return sludge were dosed continuously via peristaltic pumps. The system was operated at ambient (outdoor) temperature which varied from 24-31 °C. The food to microorganism ratio and DO were controlled in order to develop a bulking sludge dominated by filamentous microorganisms associated with suboptimal DO levels relative the the applied organic load. Target values for dissolved oxygen and F/M were 0.5 g/m^3 and 0.6 gCOD/gVSS-d, respectively. Concentration of COD in the sewage feed was increased by adding 0.2 kg/m^3 of sodium acetate.

7.2.2 Toxicity Tests

Bulking sludge was diluted with secondary effluent or concentrated by settling in order to obtain total suspended solids concentrations (TSS) of 0.25-10.0 kg/m^3. The pH of activated sludge sacrificed was 7.30. Mixed aliquots of sludge at each value of TSS were dosed with various amounts of ferrous sulfate. Sample aeration was stopped and oxygen was stripped out by nitrogen gas before toxicity tests were started. pH was adjusted to 7.6 with 0.1N NaOH after stting up the differenet solids concentrations. Sample volumes of 100 mL in 250-mL Erlenmyer flasks were amended with appropriate volumes to give desired Fe^{2+} dosages. Treated aliquots were agitated for two hours at 100 oscillations/min before assaying for dehydrogenase activity. All

tests were conducted at room temperature (22 ± 1°C).

7.2.3. Analytical Methods

SVI* (Stobbe 1964) was determined according to the procedure of Lee et al. (1983). Filamentous microorganisms were identified according to the key of Eikelboom (1975, 1977) as modified by Strom and Jenkins (1984). Identification of filamentous types was confirmed by Dr. M.G. Richard (Colorado State Univ., Fort Collins, CO). COD, TSS, and volatile suspended solids (VSS) were measured according to APHA (1985), methods 508 A, 209 D, and 209 E, respectively.

The procedures for measuring dehydrogenase activity of filamentous biomass (specific DHA) and gross biomass (gross DHA) in activated sludge followed those described by Koopman and Bitton (1987). Then, Duplicate 5.0 mL aliquots were amended with 0.5 ml 0.2% 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT). Incubation was 20 min in the dark at 20 ± 0.1°C, with periodic resuspension of sludge. The reaction was stopped by adding 0.5 mL 2.4N HCl. Specific DHA was measured by the method described in the chapter 6. Measurement of gross DHA was by extraction of INT-formazan into dimethyl sulfoxide (DMSO) and spectrophotometric determination of its concentration (see chapt. 6). Gross DHA was expressed in units of equivalent oxygen (O_2*) uptake.

Toxic inhibition data (% inhibition) were transformed using the gamma parameter (Johnson et al. 1974), where:

$$\Gamma = \frac{\% \text{ inhibition}}{100 - \% \text{ inhibition}}$$

lines of best fit to plots of Γ versus Fe^{2+} concentration were determined by least squares linear regression. The Fe^{2+} concentration at which $\Gamma = 1.0$ according the line of best fit was taken as the IC_{50}.

7.3. Results and Discussion

7.3.1. Development of Bulking Sludge

The average pilot plant conditions for 10 days of operation are given in Table 7-1. At the end of the operating period, filaments were present in "common" abundance. The dominant types were Haliscomenobacter hydrossis and Sphaerotilus natans. These types are associated with DO levels which are too low for the applied organic load (Jenkins et al. 1984). Thiothrix II, Nostocoida limicola II and type 0041 were also present in the sludge, but at lesser abundance. The work sheet used to key out the filamentous microorganisms is shown in Table 7-2.

7.3.2 Determination of IC_{50} Values

The effectiveness of ferrous sulfate was characterized in terms of the concentration required to reduce microbial DHA by 50% (IC_{50}). The procedure employed to estimate IC_{50} values is illustrated below using as an example the activated sludge at a suspended solids concentration of 0.25 kg/m^3.

Figure 7-1 (top) shows the effect of iron(II) on the filamentous component of this sludge. Specific DHA ranged from 54% at 1.25 g Fe^{2+}/m^3 to 2.5% at 12.5 g Fe^{2+}/m^3. The degree of

Table 7-1. Pilot plant operating conditions and dominant filament types[a]

Feed COD, g/m^3[b]	F/M, g COD/g VSS d	DO, g/m^3	pH	Temp., °C	Dominant filaments
314(45)	0.58(0.11)	0.61(0.2)	7.43(0.1)	31(0.64)	S. natans H. hydrossis

[a]Coefficient of variation given in ( )
[b]Sodium acetate added to sewage feed to increase COD

Table 7-2. Identification of filamentous microorganisms present in activated sludge sacrificed for toxicity teats.*

	Filament # A	B	C	D
Branching	+,False	-	-	-
Motility	-	-	-	-
Filament Shape	straight	smoothly-curved	coiled,	straight
location	ext. from floc	ext. from floc	in floc	ext. from floc
Crosswalls	+	+	+	-
Sheeth	+	-	-	+
Attached unicells	+/-	-	-	+
Filament length (μm)	100-300	50-150	50-150	20-40
diameter	1.4	0.8-1.2	1.4	0.5
Cell shape	oval-end rods	rectangles	ovals	-
size (μm)	1.4x2-3	0.8-1.2x2	1.4x1.8	-
Sulfur Deposits	-	+	-	-
Other Granules	+	+/-	+	-
Gram Stain	-	-	+	-
Neisser Stain	-	-	-	-
Commonness	common	some	some	common
Rank	2	3	4	1
Identification	Sphaerotilus natans	Thiothrix II	Nostocoida limicola	Haliscomenobacter hydrossis

*This table was provided by Dr. Michael Richard, Colorado State University.

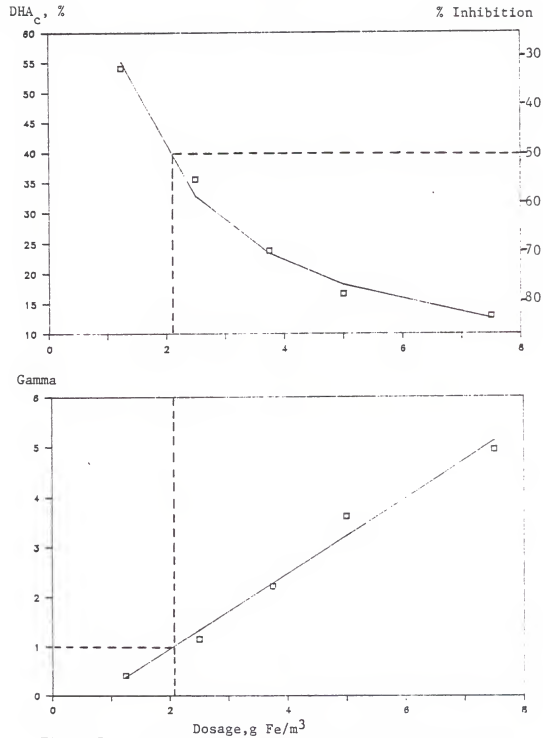


Figure 7-1. Effect of ferrous sulfate on DHA_c of bulking sludge (TSS = 0.25 kg/m^3, DHA_c of control = 76.7%). Top - relationship between DHA_c, percent inhibition and Fe^{2+} dosage. Bottom - relationship between Γ and Fe^{2+} dosage. Dashed line shows estimation of IC_{50}.

inhibition was nonlinearly related to toxicant dosage. These data were replotted in terms of at the bottom of Figure 7-1. The relationship between and iron concentration was well characterized by a linear model ($p < 0.01$; $r = 0.99$). The IC_{50} found from the line of best fit was 2.17 g/m^3.

7.3.3. Effect of Suspended Solids on Toxicity of Ferrous Sulfate to Filamentous Microorganisms in Bulking Activated Sludge

Figure 7-2 shows the influence of suspended solids on the toxicity of ferrous sulfate to filamentous microorganisms in bulking activated sludge. IC_{50} values are expressed on a volumetric basis. The chemical dosage required to inhibit 50% of specific DHA (filamentous biomass activity) increased approximately in proportion to suspended solids concentration. These data are replotted in Figure 7-3, with IC_{50} values expressed in terms of mass dosage (mg Fe^{2+}/g TSS). As shown, there was no consistent relationship exhibited between the mass based IC_{50} and the suspended solids concentration.

7.3.4 Effect of Suspended Solids on Toxicity of Ferrous Sulfate to Gross Biomass in Bulking Activated Sludge

A linear model was again applied to describe the relationship between Γ and iron concentration for the effect of iron on gross sludge biomass. The iron dose required to inhibit 50% of gross activity increased as TSS concentration increased (Figure 7-4). Gross sludge biomass was less sensitive to toxic effects than filamentous biomass at any given level of total suspended solids. IC_{50} values expressed in terms of mass dosage (mg Fe/g TSS) are

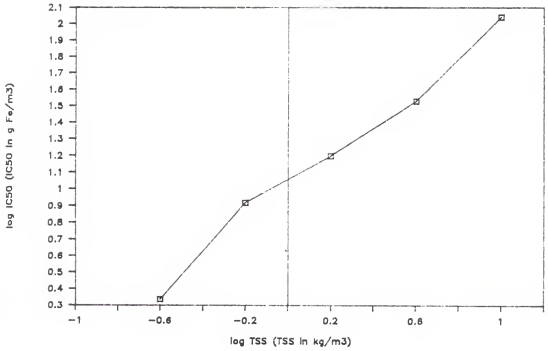


Figure 7-2. Effect of suspended solids concentration on toxicity of ferrous sulfate to filamentous microorganisms in bulking activated sludge (IC_{50}s expressed as volumetric concentrations)

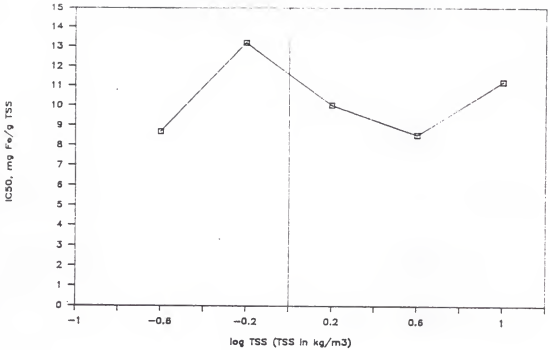


Figure 7-3. Effect of suspended solids concentration on toxicity of ferrous sulfate to filamentous microorganisms in bulking activated sludge (IC_{50}s expressed as mass Fe^{2+} per mass suspended solids).

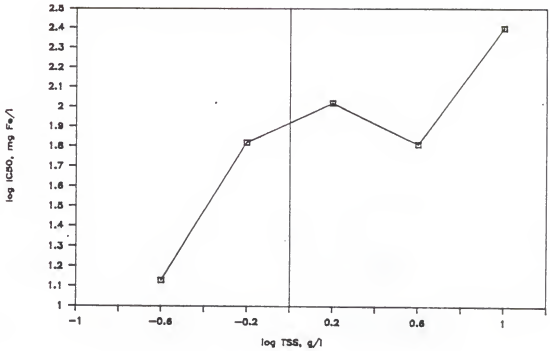


Figure 7-4. Effect of suspended solids concentration on toxicity of gross biomass in bulking activated sludge (IC_{50}s expressed as volumetric concentrations)

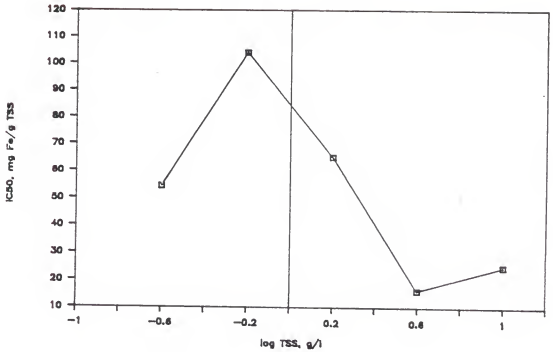


Figure 7-5. Effect of suspended solids concentrations on toxicity of gross biomass in bulking activated sludge (IC_{50}s expressed as mass Fe^{2+} per mass suspended solids)

plotted versus suspended solids concentration in Figure 7-5. There was also no consistent relationship exhibited between the mass based IC_{50} and the suspended solids concentration for gross biomass.

CHAPTER 8
SUMMARY AND CONCLUSIONS

Several aspects of the dehydrogenase assay on activated sludge and filamentous bacteria using 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride were examined. Methanol, 2+3 tetrachloroethylene/acetone, and dimethylsulfoxide were compared as solvents for extracting INT-formazan. Dimethylsulfoxide provided the most consistent extraction of INTF from activated sludge and filamentous bacteria. The relative efficiency of 2+3 tetrachloroethylene/acetone (TA) was slightly greater than that of DMSO in some cases. However, this solvent only partially extracted INTF from one of the filament types tested (type 1701) and its performance was impaired by adsorption of ferrous ion to sludge. Methanol was generally the least efficient of the solvents tested. It was particularly ineffective in extracting INTF from Fe^{2+}-treated sludges. Permeabilization of cells with lysozyme or Triton X-100 improved the INTF yields obtained with methanol. Triton X-100, the more effective of the two permeabilizing agents, increased the INTF yield obtained with methanol to the same level as that obtained with DMSO or TA. Blank corrections were significant when samples initially treated with ferrous sulfate were assayed. Two

alternative blank correction techniques were evaluated and discussed. Four different reagent solutions were tested for terminating biological INT reduction. Addition of 0.5 ml of 2.4N HCL to the 5.5-ml INT reaction mixture was the best means of terminating the enzymatic reaction, but did not prevent changes in formazan yield after prolonged storage of the reaction mixtures. It also preserved INTF formazan crystals adequately for resolution under bright field microscopy.

The validity of INT-dehydrogenase test was estabilished for assessing toxic inhibition of filamentous pure cultures (Sphaerotilus natans, Type 021N and Thiothrix) and activated sludge by ferrous sulfate. The INT dehydrogenase activity by the extraction technique (DHA_e) was correlated linearly ($p < 0.01$, a coefficient of determination (r^2) > 0.91 except $r^2 = 0.87$ in relationship between DHA_e and ATP with S. natans) with the alternative parameters (oxygen uptake and ATP). Pictures taken of samples of pure culture (Type 021N) after being exposed to ferrous sulfate and treated by INT illustrated the feasibility of INT-dehydrogenase test by counting technique (DHA_c). Excellent correlations ($r^2 > 0.95$) were found between DHA_e and DHA_c with S. natans, Type 021N and Thiothrix. The inhibitory effect of S. natans by ferrous sulfate was a function of pH and it was dramatically increased as pH was changed from 7.0 to 6.0. The order of sensitivity of pure cultures to ferrous sulfate was Thiothrix, Type 021N and S. natans (from most to least sensitive)

in terms of inhibitor concentration for 50 % reduction of activity (IC_{50}). This implies that the application of ferrous sulfate in preventing or controlling bulking problem has advantages in bulking sludge of low pH and bulking sludge caused by Thiothrix or Type 021N.

In bench-scale pilot experiments involving bulking due to Sphaerotilus natans and Thiothrix II, the INT dehydrogenase activity counting (DHA_c) technique gave an early indication of the trend in the recovery of bulking problem following ferrous sulfate treatment. Successful control of bulking was achieved by adding ferrous sulfate (one third of IC_{50}, and exposure frequency = 3.0 d^{-1}) into the aeration basin during 4-5 consecutive days until DHA_c fell below 10%. IC_{50}s for bulking sludge dominated by Sphaerotilus natans and dominated by Thiothrix II were 23-24 mg Fe/l and 12-16 g Fe/m^3, respectively. The effectiveness of ferrous sulfate for bulking control appeared to be dependent as the type of predominant filamentous organism causing the bulking. A low target value of DSVI (e.g., 200-300 mL/g) would give a successful control bulking problem by enabling more timely reversal of rising trends in DSVI and preventing losses of solids. Ferrous sulfate was more effective for bulking activated sludge caused by Thiothrix and 021N and mixed liquors of lower pH in controlling bulking. DHA_c provided a practical tool for use in monitoring and prediction of filamentous bulking.

The influence of total suspended solids concnetration on iron

toxicity to bulking activated sludge was studied. The ferrous sulfate dose on a volumetric basis required to reduce 50% of filamentous bacteria activity increased in proportion to TSS concentration. However, there was no consistent relationship exhibited between the mass based IC_{50} and the suspended solids concentration. IC_{50} values for filamentous biomass were always lower than those for gross biomass.

APPENDIX A

PROCEDURES FOR THE GRAM STAIN, MODIFIED NEISSER STAIN, INTRACELLULAR SULFUR GRANULE TEST, AND SHEATH STAIN

APPENDIX A
PROCEDURES FOR THE GRAM STAIN, MODIFIED NEISSER STAIN, INTRACELLULAR SULFUR GRANULE TEST, AND SHEATH STAIN

A.1 Gram stain (Richard et al., 1981)

A.1.1. solutions

Solution 1: ammonium oxalate crystal violet; mix A and B

A

crystal violet	2 gm
ethanol, 95%	20 ml

B

ammonium oxalate	0.8 gm
distilled water	80 ml

Solution 2: Grams modification of Lugol's solutiuon

iodine	1 gm
potassium iodide	2 gm
distilled water	300 ml

Solution 3: counterstain

safranin O	10 ml
distilled water	100 ml

A.1.2. Procedure

1. Prepare smears and air dry; do not heat fix.
2. Stain 1 min. with Solution 1; rinse gently with water.
3. Stain 1 min. with Solution 2; rinse well.
4. Hold slide at an angle and decolorize with 95% ethanol until free color has been washed off (usually takes about 30 sec). Wash with water and drain.
5. Cover smear with Solution 3 for 1 min; rinse well and blot dry.
6. Examine under oil immersion with direct illumination; red is Gram negative, blue is Gram positive.

A.2. Modified Neisser's stain (Richard et al., 1981)

A.2.1. Solutions

Solution 1:	
methylene blue	0.1 gm
ethanol, 95%	5 ml
acetic acid, glacial	5 ml
distilled water	100 ml
Solution 2:	
crystal violet, 10% in ethanol	3.3 ml
ethanol, 95%	6.7 ml
distilled water	100 ml

Mix 2 parts Solution 1 and 1 part Solution 2.

Solution 3:	
bismark brown, 1% aquous	33.3 ml
distilled water	66.7 ml

A.2.2. Procedure

1. Prepare smears and thoroughly air dry; do not heat fix.
2. Stain 30 sec. with Solution 1 and 2 mixture; rinse 1 sec.
3. Stain 1 min. with Solution 3; rinse well; blot dry.
4. Examine under oil immersion with direct illumination; yellow-brown is Neisser negative, blue-gray is Neisser positive.

A.3. Intracellular sulfur granule test (S test) (Richad et al., 1981)

Add 1 drop of sample and 1 drop of sodium sulfide solution (100 mg/L) on a microscope slide and allow to stand open to the air for 10-20 min. Place a coverslip on the preparation and gently press to exclude excess solution. Observe wet mounts at 1000X phase contrast for intracellular granules within the filaments. A positive S test is the observation of highly refractive, yellow-colored intracellular granules (sulfur granules).

A.4. Sheath stain (Richard et al., 1981)

Mix 1 drop sample and 1 drop crystal violet solution (0.1% w/v aqueous solution) on a microscope slide, cover and examine at 1000X magnification phase contrast. Cells stain deep violet while the sheaths are clear to pink.

APPENDIX B

DATA SUMMARY OF PILOT PLANT OPERATION FOR INT-DEHYDROGENASE ASSAY AS AN ADJUNCT TO BULKING CONTROL WITH FERROUS SULATE

Table B-1. Data summary of pilot plant operation for bulking control, Experiment 1

Date	SVI ml/g	DHA_e $\frac{mg\ O_2}{g\ VSS\text{-}d}$*	DHA_c %	F/M $\frac{g\ COD}{g\ VSS\text{-}d}$	Total sludge iron, mg Fe/L	Effluent filterable iron mg Fe/L
Nov. 9	106	26.6		0.32		
11	121			0.33		
13	400	28.8	86	0.44		0.3
14	320		83	0.74	19	0.3
15	1323	44.9	81	0.56	36.5	4.3
16	405	73.3	54	0.86	13	0.3
17	393	70.0	11	1.42	18	0.3
18	534	58.0	3	0.79	36	0.9
19	292	37.6	1	0.48	20	0.3
20	200	42.6	5	0.79	21	1.5
21	219	39.2	4	0.65	18	1.7
22	168	33.9	4	1.13	17	0.6
23	154	59.2	70	0.85		
24	199	46.6	83	1.33		
25	189	36.7	85	0.62		
26	179	49.3	98	0.93		0.2
27	876	56.8	96	0.48	14.9	0.2
28	477	120.5	98	1.61	19.3	0.3
29	537	119.4	95	0.83	20.2	0.1
30	621	101.7	46	0.55	17.1	1.0
Dec. 1	851	91.1	41	1.23	11.8	1.8
2	2300	83.6	81	0.66	8.0	0.6
3	529	106.3	11	0.92	28.7	0.4

Table B-2. Data summary of pilot plant operation for bulking control, Experiemnt 2

Date	SVI mL/g	DHA_e $\frac{mg\ O_2^*}{g\ VSS\text{-}d}$	DHA_c %	F/M $\frac{g\ COD}{g\ VSS\text{-}d}$	Effluent COD mg/L
Apr. 16	105	60		0.53	30
17	97	54		0.55	28
18	106	60		0.61	19
19	108	61		0.64	28
20	124	69	62	0.64	21
21	158	80	74	0.56	19
22	191	87	85	0.61	47
23	183	85	24	0.57	19
24	158	81	20	0.71	45
25	169	88	40	0.72	27
26	165	67	16	0.58	45
27	146	66	7	0.51	62
28	147	76	5	0.51	37
29	138	76	9	0.63	37
30	189	81	42	0.67	45
May 1	233	66	66	0.61	33
2	153	52	7	0.59	12
3	181	70	12	0.65	27
4	168	65	4	0.62	28
5	150	62	3	0.56	17
6	144	73	3	0.60	15
7	137	51	2	0.62	14
8	147	58	4	0.57	12
9	167	64	15	0.61	35
10	186	73		0.62	28
11	202	76		0.67	32

Table B-3. Comparison of sample-treatment methods for iron measurement

Sample	Chang (1979)[a] mg/L	Modified method[b] mg/L
Activated sludge from Nov. 14	19	19
	19	19
	20	19
	19	18
	19	19
Avg.	19.2	18.8

a 2N HCl at 100°C for 2 hrs
b Conc. HCl at room temp. (22±2°C) for one week

APPENDIX C

RAW DATA FOR THE EFFECT OF SUSPENDED SOLIDS CONCENTRATION ON TOXICITY OF FERROUS SULFATE TO BULKING ACTIVATED SLUDGE

Table C. DHA of Spharotilus natans and Thiotrix II dominant activated sludge under ferrous sulfate inhibition depending on MLSS

MLSS g/l	Dosage Fe mg/gTSS	DHA_e mg O_2*/g VSS-d	DHA_c % Active cells
0.25	0	54.9	76.7
	5	54.6	54.1
	10	56.0	35.6
	15	51.5	23.8
	20	51.9	16.6
	30	47.1	12.9
	40	27.8	8.2
	50	29.7	2.5
0.63	0	64.4	70.5
	5	58.1	51.7
	10	58.9	47.2
	15	49.1	33.3
	20	47.8	25.4
	30	49.1	21.1
	40	48.7	17.2
	50	40.5	15.2
1.60	0	44.9	81.4
	5	32.4	47.0
	10	32.5	43.3
	15	30.1	47.4
	20	29.5	20.3
	30	29.1	12.8
	40	26.2	8.7
	50	23.8	7.4
4.00	0	42.8	77.2
	5	32.3	40.2
	10	22.0	37.1
	15	21.7	32.4
	20	20.2	9.6
	30	19.6	7.5
	40	17.4	5.2
	50	15.6	4.0
10.0	0	57.2	65.0
	5	54.3	59.4
	10	57.8	48.6
	15	57.6	25.4
	20	40.5	15.5
	30	22.0	7.4
	40	20.1	4.2
	50	18.2	3.5

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BIOGRAPHICAL SKETCH

Chan-Won Lee, son of Sang-Young and Keun-Oak, was born on January 17, 1954, in Pusan, Korea. In March, 1973, he entered Seoul National University, Seoul, Korea, and received his bachelor's degree in food science and technology in February 1977. He enrolled in graduate school in March 1977 and was appointed as a teaching assistant for the courses of food microbiology and industrial fermentation technology. He was awarded a Master of Science degree in food science and technology in February 1979. His thesis was entitled "Studies on the Flavor Components of Apple Fine Distillates Aged with Korean Oak Chips." Upon graduation, he served for six months in the army and then joined the R&D department in the Dong-Suh food company as a researcher. In March, 1980, he was appointed full-time lecturer in Kung-Nam College in charge of undergraduate courses of unit operation and analytical chemistry. He entered the University of Florida in August 1981 and received his Master of Agriculture in food science and human nutrition in December 1983. He transfered to the Department of Environmental Engineering Sciences in January 1984, majoring in water pollution control. He married Soon-Hae, daughter of Dong-Wan and Sang-Suk Kim, in August 1981. He has two

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ben Koopman, Chairman
Associate Professor of Environmental Engineering Sciences

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Gabriel Bitton
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